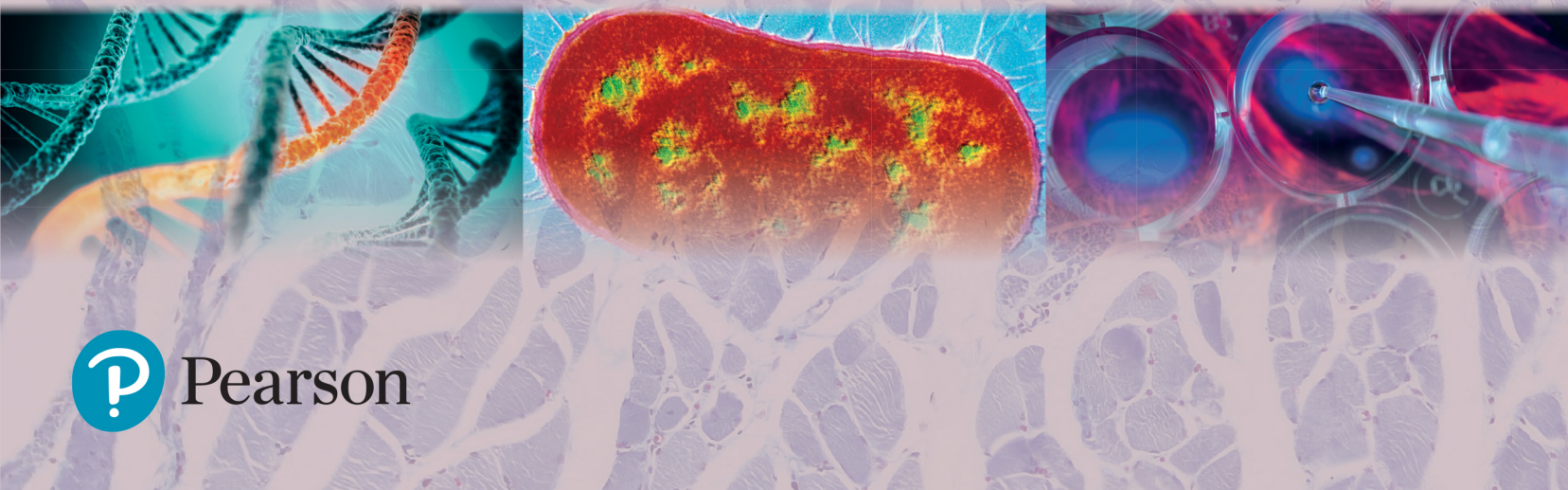




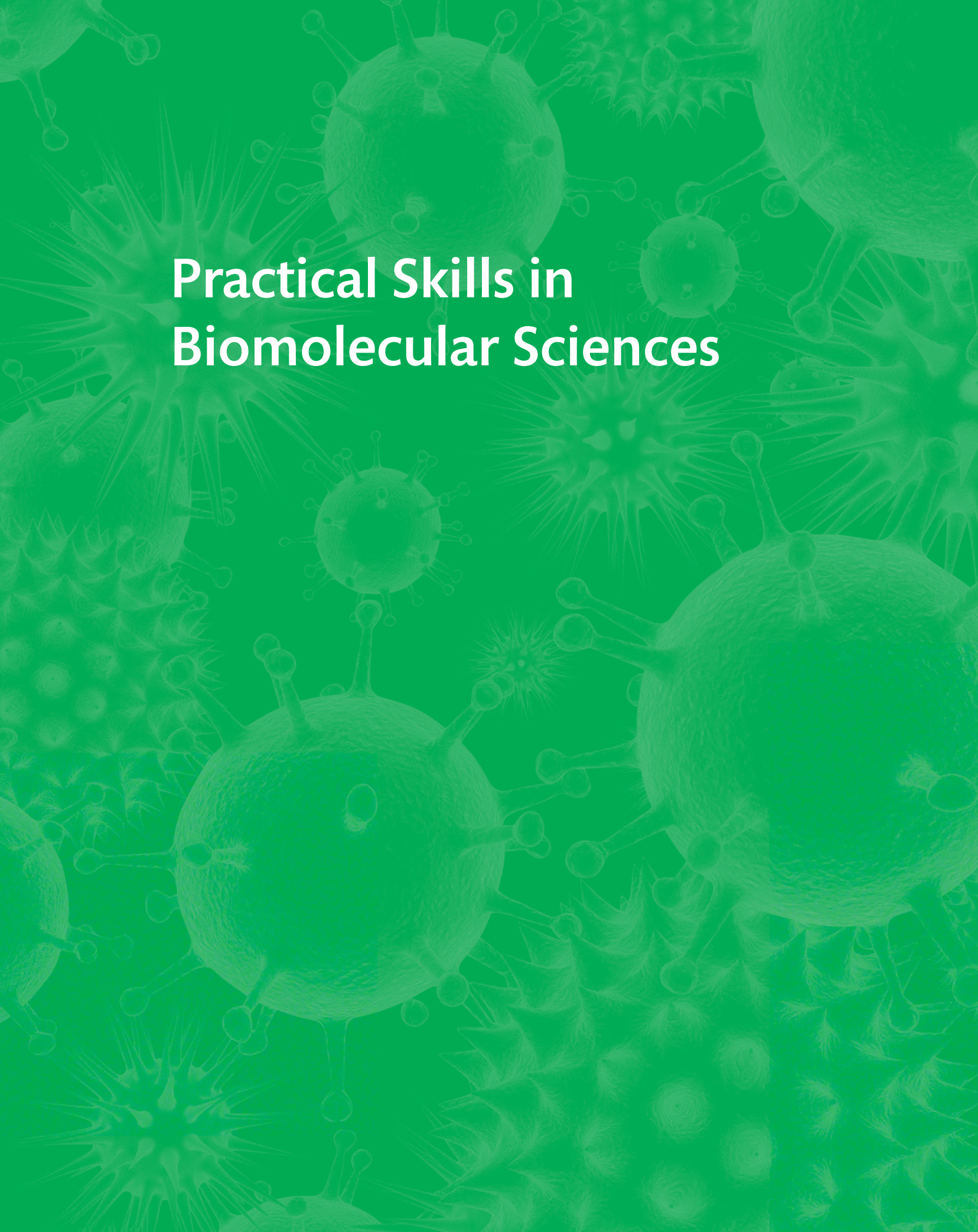
Rob Reed
David Holmes
Jonathan Weyers
Allan Jones

Practical Skills in BIOMOLECULAR SCIENCES

Sixth Edition



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The background of the image is a dense, monochromatic green field filled with various biological structures. These include spherical viruses with prominent spikes (resembling coronaviruses), smaller spherical particles, and elongated, filamentous structures. The overall effect is a complex, textured pattern that suggests a microscopic or molecular environment.

Practical Skills in Biomolecular Sciences



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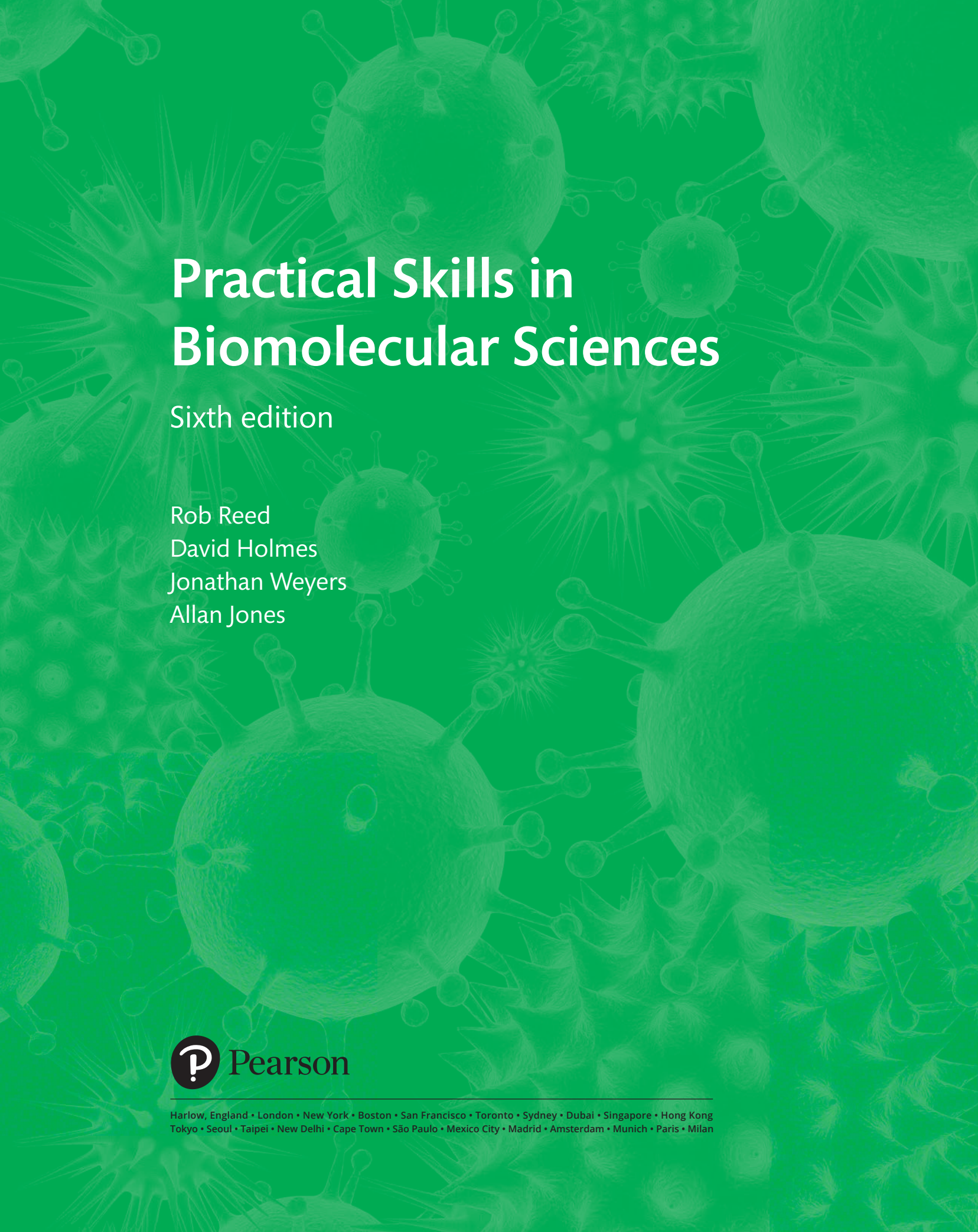
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Practical Skills in Biomolecular Sciences

Sixth edition

Rob Reed
David Holmes
Jonathan Weyers
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Companion Website

For open-access **student resources** specifically written to complement this textbook and support your learning, please visit go.pearson.com/uk/he/resources



Lecturer Resources

For password-protected online resources tailored to support the use of this textbook in teaching, please visit go.pearson.com/uk/he/resources

Preface to the 6th edition

The primary aim of this revision of *Practical Skills in Biomolecular Sciences* was to update the text, but we also wished to respond to the helpful comments of several anonymous reviewers of the 5th edition, and in so doing, to reorganise the chapters and include significant new material. The main structural changes we have made are to (1) reorder and rewrite several chapters in the first two sections; (2) add three new chapters on working with bacteria, eukaryotic microbes and viruses; and (3) revise the material on use of software and online sources, to reflect the greater level of knowledge and experience of today's students. In terms of the text itself, we have sought to use more positive phrasing throughout, to emphasise the active nature of learning in this discipline. Some details of further changes and additions are listed on the back cover. The text references and sources for further study have been updated, while the popular study exercises have been retained.

We thank everyone who helped us with earlier editions, and for this one acknowledge in particular the assistance of Professor Gary Black, for feedback on the chapters dealing with molecular genetics, Jill Muller of CQUniversity in helping to revise the material on finding and citing sources, Lou Attwood for her work in copy editing the text, and Indrasena Mukhopadhyay and Nikhil Kumar in coordinating the production of the text and images, together with other staff who were involved in the book's production. We also recognise Rufus Curnow for his enduring support of all of the *Practical Skills* titles. Although this revision has largely been the work of two of the original authors (RHR and JDBW), we thank Allan Jones and Dave Holmes for their contributions to the *Practical Skills* series throughout the years. Finally, we thank staff at all institutions who have adopted this text. Practical education across the life sciences has come under increasing pressure in recent years, with diminishing resources and timetabling allocation. Yet such

changes cannot alter the fundamental fact that subjects such as biochemistry, biomedical sciences, genetics, microbiology and molecular biology are practical in nature, where students learn most effectively through 'hands-on' experience in the lab and the field. We hope that this book will help students to prepare better for practicals, projects, lectures, seminars, examinations and assignments, to gain greater enjoyment from taking part in them and to learn more about the nature of living systems at the cellular and molecular level.

The book is divided into several sections:

- **Chapters 1–8 cover general skills**, including self-management and personal development; how to learn; teamwork; and how to locate, evaluate and cite sources.
- **Chapters 9–18 deal with assessment**, including written assignments; practicals and projects; oral and poster presentations; revision and examinations.
- **Chapters 19–70 cover a broad range of specific practical skills and analytical techniques**, ranging from basic laboratory procedures to more advanced techniques.
- **Chapters 71–77 explain data analysis and presentation**, ranging from the presentation of results as graphs or tables through to the application of statistical tests, with worked examples.
- **Study exercises and problems** are provided for each chapter. They enable you to check your understanding and to practice key calculations, either on your own, under the guidance of a tutor, or working with other students. Numerical and text-based answers are provided at the book's website at: go.pearson.com/uk/he/resources.

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April 2021

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List of abbreviations

A	absorbance (e.g. A_{260} = absorbance at 260 nm)	IRMA	immunoradiometric assay
AC	affinity chromatography	IRMS	isotope ratio mass spectroscopy
ACDP	Advisory Committee on Dangerous Pathogens	ISE	ion-selective electrode
ADP	adenosine diphosphate	K_m	Michaelis constant
ANOVA	analysis of variance	K_w	ionisation constant of water
ATP	adenosine triphosphate	LCB	lactophenol cotton blue
BSA	bovine serum albumin	LDH	lactate dehydrogenase
CCCP	carbonylcyanide <i>m</i> -chlorophenylhydrazone	LSD	least significant difference
CE	capillary electrophoresis	MEKC	micellar electrokinetic chromatography
CFU	colony-forming unit	MPN	most probable number
CGE	capillary gel electrophoresis	M_r	relative molecular mass
COSHH	Control of Substances Hazardous to Health	MRI	magnetic resonance imaging
COVID	coronavirus disease	MS	mass spectrometry
CRISPR-Cas	clustered regularly interspaced short palindromic repeat-CRISPR-associated	NAD^+	nicotinamide adenine dinucleotide (oxidised form)
CTP	cytosine triphosphate	NADH	nicotinamide adenine dinucleotide (reduced form)
CZE	capillary zone electrophoresis	$NADP^+$	nicotinamide adenine dinucleotide phosphate -(oxidised form)
ddNTP	dideoxynucleotide triphosphate	NADPH	nicotinamide adenine dinucleotide phosphate -(reduced form)
DMSO	dimethyl sulfoxide	NH	null hypothesis
DNA	deoxyribonucleic acid	NMR	nuclear magnetic resonance
dNTP	deoxyribonucleoside triphosphate	PAGE	polyacrylamide gel electrophoresis
d.p.m.	disintegrations per minute	PAR	photosynthetically active radiation
dsDNA	double-stranded DNA	PCR	polymerase chain reaction
ECD	electron capture detector	PDP	personal development planning
EDTA	ethylenediaminetetraacetic acid	PEG	polyethylene glycol
EI	electron impact ionisation	PFD	photon flux density
EIA	enzyme immunoassay	PFGE	pulsed field gel electrophoresis
ELISA	enzyme-linked immunosorbent assay	PFU	plaque-forming unit
EMR	electromagnetic radiation	pH	$-\log_{10}$ proton concentration (activity), in mol l^{-1}
EOF	electro-osmotic flow	PI	photosynthetic irradiance
ESR	electron spin resonance	PPFD	photosynthetic photon flux density
F	Faraday constant	PPi	pyrophosphate (inorganic)
FIA	fluorescence immunoassay	PVA	polyvinyl alcohol
FID	flame ionisation detector	PY-MS	pyrolysis-mass spectrometry
FITC	fluorescein isothiocyanate	R	universal gas constant
FPLC	fast protein liquid chromatography	RCF	relative centrifugal field
FT	Fourier transformation	R_f	relative frontal mobility
g	acceleration due to gravity	RIA	radioimmunoassay
GC	gas chromatography	RID	radioimmunodiffusion
GPC	gel permeation chromatography	RNA	ribonucleic acid
HEPES	<i>N</i> -[2-hydroxyethyl]piperazine- <i>N'</i> - [ethanesulfonic acid]	RP-HPLC	reverse phase high-performance liquid - chromatography
HIC	hydrophobic interaction chromatography	r.p.m.	revolutions per minute
HPLC	high-performance liquid chromatography	RT	reverse transcriptase
IEC	ion-exchange chromatography	SARS-CoV	severe acute respiratory syndrome-coronavirus
IEF	isoelectric focusing	SDS	sodium dodecyl sulfate
Ig	immunoglobulin		
IMAC	immobilised metal affinity chromatography		
IR	infrared (radiation)		
IRGA	infrared gas analyser		

SE	standard error (of the sample mean)	TLC	thin-layer chromatography
SEM	scanning electron microscopy	TRIS	tris(hydroxymethyl)aminomethane
SI	Système International d'Unités	TTP	thymidine triphosphate
ssRNA	single-stranded RNA	UNG	uracil- <i>N</i> -glycosylase
STP	standard temperature and pressure	URL	uniform resource locator
TCA	trichloroacetic acid	UV	ultraviolet (radiation)
TCD	thermal conductivity detector	V_{\max}	maximum velocity
TEM	transmission electron microscopy	<i>z</i>	net charge on an ion
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine		



Study strategies

1. Developing your skills	3
2. Self-management	7
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4. Making the most of learning resources	26
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1 Developing your skills

Using skills terminology – different phrases may be used to describe skills and associated personal qualities, depending on place or context. These include: ‘graduate attributes’, ‘transferable skills’, ‘personal transferable skills’ (PTS), ‘key skills’, ‘core skills’ and ‘competences’.

A degree in the biomolecular sciences covers both knowledge and skills. Both are integral to your future qualification: along with your personal attributes, they are what your future employer will be looking for when they hire you. There are many possible classifications of skills and your university may favour a specific approach. One possible division relevant to all biological sciences is into generic and practical skills (Fig. 1.1).

Generic skills are, by definition, those applicable in a range of study and work scenarios. For example, self-management, cognitive (thinking) and interpersonal skills are central to the notion of university education and becoming a graduate. Practical skills have a hands-on aspect and a direct relevance to study, research and employment in the biomolecular sciences. For example, the skills involved in measuring pH (Chapter 24) are relevant at all levels, in fields as diverse as cell and tissue culture, assaying metabolites and molecular genetics.

The phrase ‘Practical Skills’ in the title of this book implies a focus on laboratory skills, and the text covers a broad range of these in some detail. However, many generic skills are also covered, because they too are essential in any future career as a biomolecular scientist. A good understanding of these skills will help you to succeed and to place your studies within a wider context.

KEY POINT All biomolecular sciences are essentially practical subjects, and therefore involve highly developed laboratory skills. The importance that your lecturers place on practical skills will probably be evident from the large proportion of time you will spend on practical work in your course.

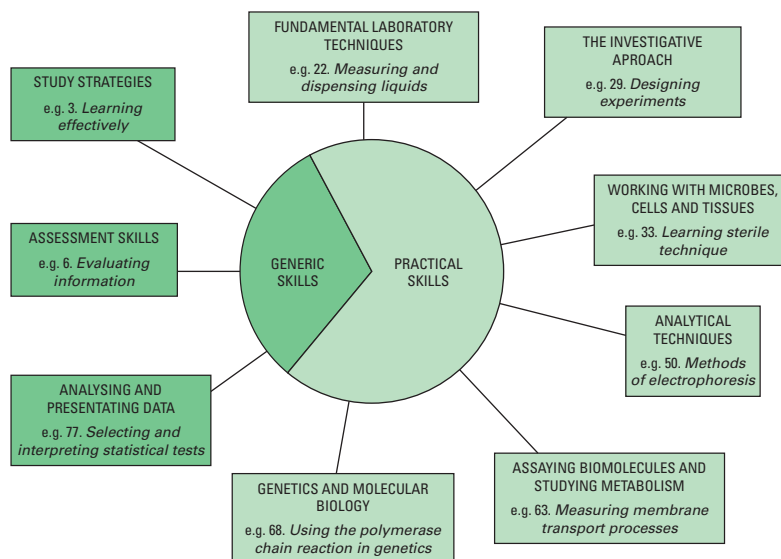


Fig. 1.1 An outline of skills relevant to biomolecular scientists, as covered in this textbook. The skill sub-categories (rectangular boxes) on each side correspond to the book's section headings, and representative chapter titles are shown for each section. Generic skills are mainly covered in the first two sections and in the last section (a total of 25 chapters), whereas the bulk of the book (comprising six sections and 52 chapters) covers practical skills. For the full section and chapter listing, see pp. v–vii.

Finding out about the skills covered in your studies – programme (degree, course) and module (unit) handbooks for your subject will draw attention to the skills elements of your course. Usually the learning outcomes (objectives) will summarise the skills that are covered (Chapter 3). While the precise topics and related skills covered in lectures and practicals will reflect the interests, expertise and experience of lecturers, the curriculum must also adhere to national standards. In the UK, these are laid out in a 'Subject Benchmark Statement' published by the Quality Assurance Agency for Higher Education and they are inspected through rigorous institutional and programme review procedures. Appendix 1 provides a listing of skills from this Benchmark Statement for the Biosciences, showing where these are covered in this book.

Example

The skills involved in teamwork cannot be developed fully without a deeper understanding of the interrelationships involved in successful groups. The context will be different for every group and a flexible approach will always be required, according to the individuals involved and the nature of the task.

Taking opportunities to develop and practise skills in your private or social life – you could, for example, practise spreadsheet skills by organising personal or club finances using Microsoft *Excel*, or teamwork skills within any university clubs or societies you may join.

Identifying the range of skills relevant to biomolecular sciences

The biomolecular sciences cover a wide range of topics, including studies on whole organisms, cells and molecules. Accordingly, the range of skills involved is extremely large. To accommodate this diversity, this book is divided into nine sections dealing with related skill areas (Fig. 1.1) with a total of 77 chapters, each covering a different topic in detail.

The word 'skill' implies much more than the robotic learning of, for example, a laboratory routine. Of course, some of the tasks you will be asked to carry out in practical classes will be repetitive. Certain techniques require manual dexterity and attention to detail if accuracy and precision are to be attained, and the necessary competence often requires practice to make perfect. However, a deeper understanding of the context of a technique is important if the skill is to be appreciated fully and then transferred to a new situation. That is why this text is not simply a 'recipe book' of methods and why it includes background information, tips and worked examples, as well as study exercises to test your understanding.

Reflecting on the transferability of skills

The term 'transferability' is often used in relation to skills to imply that someone with knowledge, understanding or ability gained in one situation can adapt or extend this for application in a different context. In some cases, the transfer of a skill is immediately obvious. Take, for example, the ability to use a spreadsheet to summarise experimental data and create a graph to illustrate results. Once the key concepts and commands are learned (Chapter 72), they can be applied to many instances outside the biomolecular sciences where this type of output is used. This is not only true for similar data sets, but also in unrelated situations, such as making up a financial balance sheet and creating a pie chart to show sources of expenditure. Similarly, knowing the requirements for good tabulation and graph drawing (Chapters 73 and 74), perhaps practised by hand in earlier work, might help you use spreadsheet commands to make the output suit your needs.

Other cases may be less clear but equally valid. For example, towards the end of your undergraduate studies you may be involved in designing experiments as part of your project work. This task will draw on several skills gained at earlier stages in your course, such as preparing solutions (Chapter 23), deciding about numbers of replicates and experimental layout (Chapter 29) and perhaps carrying out some particular analytical method. (Chapters 43–70). How and when might you transfer this complex set of skills? In the workplace, it is unlikely that you would be asked to repeat exactly the same process, but in critically evaluating a problem or in planning a complex project for a new employer, you will need to use many of the time-management, organisational and analytical skills developed when designing and carrying out experiments. The same applies to information retrieval and evaluation and writing essays and dissertations, when transferred to the task of analysing or writing a business report.

Making the most of your graduate attributes

The skills emphasised in biomolecular sciences courses are sometimes considered alongside a university-wide framework of graduate attributes that are intended to summarise the qualities and skills that an employer might expect in those with qualifications from your institution. The associated

Definitions

Employability – the ability to secure employment and follow a long-term career, requiring: (1) a mix of subject knowledge and understanding; (2) the possession of relevant practical and generic skills; (3) suitable personal attributes and attitudes; (4) an appreciation of workplace values; and (5) an understanding of the need for continuing personal and professional development.

Graduate attributes – the set of qualities and skills that graduates develop through their academic study and engagement in student life, including the acquisition of subject-specific knowledge, intellectual skills, practical skills, personal skills and digital literacy.

notion of ‘graduateness’ summarises the effect of degree-level experience and learning on an individual. This in turn is connected with the concept of ‘employability’ which encompasses those skills and qualities required to gain and maintain employment. An understanding of these concepts is important for every student, as this not only leads to a better appreciation of the value of certain activities and assessments, but also provides a specialised vocabulary and gives insights about your personal development and career potential.

At the end of your course, which may be some time away, you will aim to get a job and start on your chosen career path. You will need to sell yourself to your future employer, firstly in your application form and curriculum vitae (Chapter 8), and perhaps later at interview. Companies rarely employ bioscience graduates simply because they know how to carry out a particular lab routine or because they can recall specific facts about their chosen degree subject. In addition to subject expertise, they will be looking for a range of graduate-level skills and attributes. Typically, for example, they will seek employees who can demonstrate the ability to work in a team, to speak effectively and write clearly about their work. All of these skills and attributes can be developed at different stages during your university studies.

KEY POINT Factual knowledge can be important in degrees with a strong vocational element, but understanding how to find and evaluate information is often rated more highly by employers than the ability to memorise facts.

Most likely, your future employer(s) will seek someone with an organised yet flexible mind, capable of demonstrating a logical approach to problems – someone who has a range of skills and who can transfer these skills to new situations. Many competing applicants will probably have similar qualifications. If you want the job, you will have to show that your additional skills and personal attributes place you above the other candidates.

Text reference

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STUDY EXERCISES

1.1 Evaluate your skills. Examine the list of skill topics shown in the chapter listing on pp. v–vii. Now create a new table with two columns, like the one on the right-hand side. The first half of this table should indicate *five* skills you feel confident about in column 1 and show where you demonstrated this skill in column 2 (for example, ‘working in a team’ and ‘in a first year group project on marine biology’). The second half of the table should show *five* skills you do not feel confident about, or you recognise need development (for example, communicating in verbal form). List these in column 1 and in column 2 list ways in which you think the course material and activities in your current modules will provide you with the opportunity to develop these skills.

1.2 Find skills resources. For at least one of the skills in the second half of the table in Study exercise 1.1, check your university’s library database to see if there are any texts on that subject. Borrow an appropriate book and read the relevant sections. Alternatively, carry out a search for relevant websites (there are many); decide which are useful and bookmark them for future use.

Skills I feel confident about	Where demonstrated
1.	
2.	
3.	
4.	
5.	
Skills that I could develop	Opportunities for development
6.	
7.	
8.	
9.	
10.	

1.3 Analyse your goals and aspirations. Spend a little time thinking what you hope to gain from university. See if your friends have the same aspirations. Think about and/or discuss how these goals can be achieved, while keeping the necessary balance between university work, paid employment and your social life.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

2 Self-management

Learning how to learn – this is a vital self-management skill for university and beyond, covered in Chapter 3.

Definitions

Aim (or Goal) – a long-term achievement you want to work towards, reflecting your ambitions.

Milestone – a ‘marker’ or key event along the way towards an objective or aim.

Objective – a well-defined short-term step towards your overall aim.

Outcome (or Target) – a measurable result from your activities.

Note: authorities differ on the definition of these terms, often depending on context. The above definitions relate to self-management.

Using the ‘SMART’ approach to write objectives. You should ensure they are:

- **Specific** – clear and unambiguous
- **Measurable** – with quantified targets, so you can assess progress
- **Achievable** – attainable within your abilities and resources
- **Relevant** – helping to achieve your overall aim
- **Timed** – so you can work to a suitable timeline for completion

The best objectives will, therefore, have detail on aspects of ‘what’, ‘how’, ‘where’ and ‘when’. The objective ‘to spend an extra hour each week on directed study in microbiology next term’ addresses these aspects and fulfils the SMART criteria, in contrast to a general intention ‘to study more’.

The term ‘self-management’ covers a wide range of skills, from being able to organise your life to understanding how to learn independently, making up an important subset of the broader skills highlighted in Chapter 1. This will become increasingly important throughout your studies, until you reach a stage when you will be expected to be able to work independently –for example, when completing a research project in your final year.

KEY POINT Being able to self-manage is a key characteristic of a successful graduate and being able to demonstrate this is a strong aid to employability (Chapters 1 and 8).

Planning your approach to university

As in all things, you are likely to achieve more from your time at university if you make purposeful decisions about what you want and how you are going to get there. Thinking about exactly what you hope to accomplish in your studies and personal life will help you to plan and prioritise your activities and make the most of your time. Typically, this will involve the following steps:

1. **Establishing your aims.** These are the broad statements of intent, setting out the end results you would like to achieve. They are sometimes called goals. You need to consider what you wish to accomplish by the end of your studies, for example, ‘graduate with a good degree’, as well as the other things you’d like to achieve during your time at university, such as ‘learn Spanish’.
2. **Setting your objectives.** These are more specific in their focus, when compared with aims. In essence, they are the smaller steps that help you to achieve an overall aim, for example, ‘improve my ability to use spreadsheets’. Each objective should also have a measurable outcome (a target).

The process of writing out your aims and specifying your objectives is an iterative one. Both should be reconsidered from time to time to ensure their relevance. Also, once you’ve completed any particular objective, you should re-evaluate your plans, and, where necessary, establish new ones.

Once you have drafted your aims, it may help to discuss them with a friend or tutor. Then write out some objectives within different time frames, forming an overall plan. Make sure that your objectives fit the ‘SMART’ criteria.

KEY POINT There are no hard-and-fast rules around setting out your aims, writing objectives and planning – some people prefer a highly structured approach while others follow a looser path. Self-management involves deciding what works best for you, thereby making the most of your time.

Example

A possible set of objectives for a 1st year biomolecular sciences student:

Short term

- Allocate two hours on Monday, Wednesday and Thursday to research my biochemistry essay.
- Join the tennis club before mid-term.

Medium term

- Set up a revision timetable for the end of semester exam by week 5.
- Find out about volunteering at the local food bank at the start of Semester 2.

Longer term

- Improve my exam skills by attending the learning centre's sessions in Semester 3.
- Discuss with my flatmates options for accommodation next year and search for options.

Using formal organisational systems – those produced by e.g. Filofax, Time Manager International (TMI) or Day-Timer tend to be aimed at the business market, and are often relatively expensive.

Planning complex projects – longer, multipart tasks like research projects (Chapter 32) may benefit from a more organised approach, where you track your progress in elements of the work, taking account of their interdependence and your achievement of relevant milestones. This can be organised and monitored by creating a specialised 'Gantt' chart with the elements displayed on the vertical axis and time (and progress) on the horizontal axis (Chapter 32).

Keeping your decluttering efforts under control – work quickly and effectively on organising your workspace; don't use tidying up as a form of work-avoidance (procrastination, p. 12).

Organising your time and tasks

Being better organised should help you to achieve more. This applies to things such as the tidiness of your desk and the filing of your notes, but also to the focus of your efforts in achieving your objectives. Smartphone calendars allow you to organise your activities and can be used to provide reminders for important activities. As well as these, many more specialised time-management and productivity apps are available (search 'time management [or planner] apps for students'), most of which will sync with your smartphone calendar. Alternatively, you may prefer to use a 'planner' type of diary. Apps and diary-style planners are convenient for recording notes and 'to-do' lists, while post-it notes are a low-tech method of writing down short-term lists of tasks.

A revision timetable (Chapter 17) is a good example of a short-term plan – and a similar approach can be taken to make the most of your time in other situations.

- **For all assignments such as essays and reports, enter submission dates in your diary/planner** – work back in time to the present, entering milestone tasks (see, for example, Fig. 32.2) so that you can complete the work on time (for example, 'complete literature survey by now'). Put reminders in your smartphone in advance of each milestone, ensuring that you give yourself enough time to complete the task.
- **Note down the times of all lectures, tutorials, lab classes and other commitments.**
- **Consult these entries to plan out the forthcoming week or month.**
- **Write down your daily and weekly 'to do' checklists** – keep them in one place.
- **Use your checklists to monitor progress on large tasks** – recognise that you don't necessarily have to complete the component parts in a linear sequence; for example, when writing a project report (Chapter 12).

Workspace organisation is another aspect where people have their own preferences: for example, you might be a person who tidies up as you go, or you might 'declutter' each week or before each major new task. Reflect on your current approach and decide if it needs to be improved, and how this might be achieved. Useful tips include:

- **ensure you have enough folders/ring-binders** – organise the paperwork for modules on your course using these
- **set out your bookshelves so you know where to find key textbooks and sources** – keep them from cluttering your desk when not being used
- **ahead of a big task, make sure you have sufficient paper, pens, printer ink, etc.** so you aren't interrupted by not having these items when needed
- **make sure your working environment is reasonably quiet** – make sure the temperature is suitable, and includes room to spread out sources, space for your computer or laptop and good lighting
- **decide how you will reward yourself for achieving milestones and objectives** – this can be as simple as deciding that you will take some exercise or eat a snack.

Non-academic activities will continue to be important while you are working on a big academic task, which is another reason for good planning.

Examples might include organising shopping for food, clothes, etc., or creating, tracking and sticking to a financial budget. Try to timetable these activities for periods when you feel less inclined to study.

Re-balancing your activities

If you feel that the balance of your working and personal time isn't right, then you could try analysing your current activities, since this is an important factor in being a successful student. However, it is important to recognise that putting time-management techniques into practice is an individual matter: self-discipline and self-evaluation, based on what works for you, will enable you to develop effective working systems.

Analysing and organising your time

Knowledge of how you currently spend your time is key to developing better time-management. To do this, you might keep a log of your different activities over, say, a week. Ideally, this will cover the full range of your activities, so that it is representative. You might, for example, create a table divided into half-hour segments for each day, and note down what you do during each time slot. Alternatively, you could try to recreate a past record of the past few days from memory, though this is likely to be less accurate.

Think beforehand how you should categorise the different things that you do while awake, from the mundane (for example, cooking and eating) to the timetabled (for example, lectures and practicals). For example, it might be relevant to group your activities under broad terms like 'committed time', 'maintenance time' and 'discretionary'. You then could use a spreadsheet (Chapter 72) to summarise this information and present it as, say, a pie chart or a bar chart (p. 575–576), so you can visualise the relative proportions. You can now analyse your data, seeking answers to questions such as:

- How much time do I spend on each category in a typical week?
- How do I divide my time between study, general activities and relaxation?
- Am I satisfied with these time allocations, when compared to the importance of each category?
- How much of my time is used effectively?
- What changes do I want to make?

The key overarching question for you to answer is: **'Does my present allocation to committed time allow me to fulfil my aims?'**

Analysing and organising your tasks

Not only do you need to decide on your allocation of time across different categories of activity, you need to choose what to do and when. One way of achieving this is to analyse your current tasks according to their importance and urgency (Fig. 2.1). Important tasks are those with significant consequences, such as studying for a test whose results will impact on your final grade, while urgent tasks are those which must be done as a top priority and at short notice, such as work towards an impending essay deadline. One approach is to allocate items on your checklist of current tasks according to their position on a grid showing relative urgency and importance (Fig. 2.1a). Then, organise your tasks by prioritising those in the 'urgent and important' category and downgrading those in the 'non-urgent and unimportant' category, ranking the 'important' and 'urgent' ones as you see fit.

Definitions

Committed time – timetabled activities involving your academic objectives, including lectures, practicals, tutorials, work on assessments and associated personal study/revision.

Maintenance time – that spent supporting your general activities, such as shopping, cleaning and laundry.

Discretionary time – time for you to use as you wish, for example, recreation, sport, hobbies and socialising.

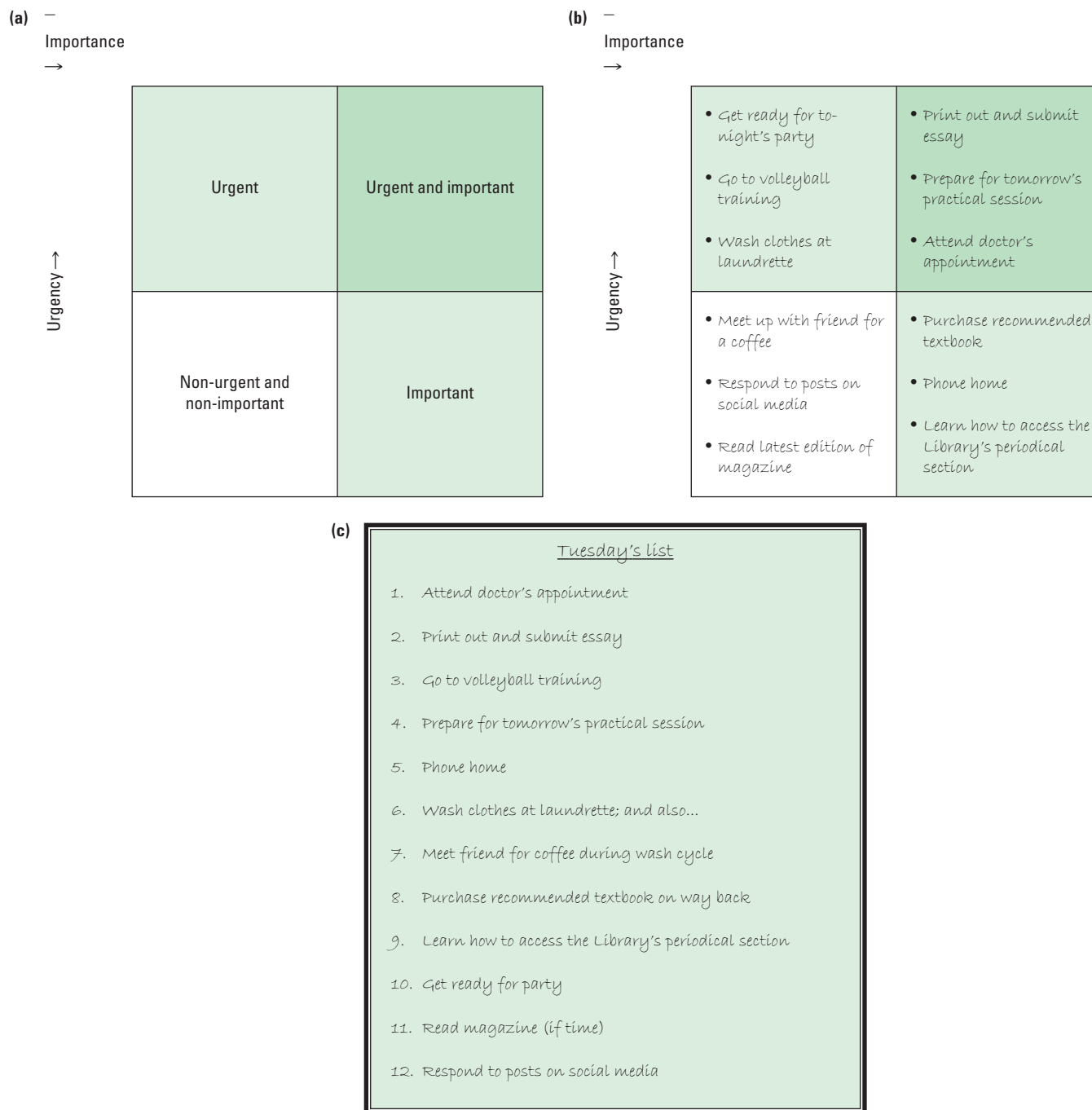


Fig. 2.1 How to prioritise your activities for a checklist. (a) Suggested layout for prioritising activities. (b) The grid from (a), populated with some example activities. Note that each person's categorisation might be slightly different. (c) The resulting checklist of priorities arising from this approach. Again, although the broad results will be similar, each person's list might differ in detail, especially in the middle section.

A disadvantage of this approach is that it may lead you to focus on impending one-off activities that you judge to be important and urgent, compared with tasks that are more routine or long-term, or discretionary. For example, you might prioritise preparing for an end-of week presentation over your regular sports sessions, or ahead of learning a language. The

Using checklists as often as possible – post your lists in places where they are easily visible and frequently seen, such as on a pin-board near to your desk. Ticking items off your checklist as they are completed gives you a feeling of accomplishment and progress, reinforcing your motivation.

Understanding the benefits of consciously managing your time – these include:

- greater control over your activities
- improved productivity
- improved performance
- less guilt about timetabled recreation
- enhanced mental well-being – avoidance of stress.

latter type of ‘elephant task’ is best managed by scheduling frequent small ‘bites’ over a longer time period, and making sure that they are allocated sufficient time. Tasks will often change in their rating through time: that distant important but not urgent project report with a submission date at the end of semester will become progressively more urgent as the deadline approaches.

If it suits your approach, make a weekly plan (Fig. 2.2) incorporating all of your routine activities and then fill the gaps with the less regular tasks, prioritised as discussed above. This might be supplemented by daily checklists. All your plans and checklists should be flexible, but should form the basis of most of your activities unless your circumstances change. Also, the time you take to review and plan needs to be scheduled as a brief daily activity. Box 2.1 provides further tips for effective planning and working.

KEY POINT At the end of each day, review the success of that day’s checklist and create a new one for the following day. Add in new tasks and carry over previous ones as appropriate, deciding the priority for each. It’s a good idea to put the most ‘urgent and important’ tasks earlier in the day so they stand a better chance of being completed.

WEEKLY DIARY

Week beginning:

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
DATE							

7–8 am		Breakfast	Breakfast	Breakfast	Breakfast	Breakfast	
8–9		Preparation	Preparation	Preparation	Preparation	Preparation	Breakfast
9–10	Breakfast	PE112(L)	PE112(L)	PE112(L)	PE112(L)	BIOL(P)	Travel
10–11	FREE	CHEM(L)	CHEM(L)	CHEM(L)	CHEM(L)	BIOL(P)	WORK
11–12	STUDY	STUDY	STUDY	STUDY	STUDY	BIOL(P)	WORK
12–1 pm	STUDY	BIOL(L)	BIOL(L)	BIOL(L)	BIOL(L)	TUTORIAL	WORK
1–2	Lunch	Lunch	Lunch	Lunch	Lunch	Lunch	Lunch
2–3	(VOLLEY-	CHEM(P)	STUDY	SPORT	PE112(P)	STUDY	WORK
3–4	BALL	CHEM(P)	STUDY	(VOLLEY-	PE112(P)	STUDY	WORK
4–5	MATCH)	CHEM(P)	STUDY	BALL	PE112(P)	SHOPPING	WORK
5–6	FREE	STUDY	STUDY	CLUB)	STUDY	TEA ROTA	WORK
6–7	Tea	Tea	Tea	Tea	Tea	Tea	Tea
7–8	FREE*	STUDY	STUDY	FREE*	STUDY	FREE*	FREE
8–9	FREE*	STUDY	STUDY	FREE*	STUDY	FREE*	FREE
9–10	FREE*	FREE*	STUDY	FREE*	STUDY	FREE*	FREE

Study (h)	2	10	11	4	11	6	0
Other (h)	13	5	4	11	4	9	15

Total study time = 44 h

Fig. 2.2 A weekly diary with an example of entries for a first-year science student with a Saturday job and active membership of a volleyball club. Note that ‘free time’ can change to ‘study time’, in sessions marked with an asterisk when work or exam revision requires the extra effort. Study time (including attendance at lectures, practicals and tutorials) thus represents between 42 and 50% of the total time.

Box 2.1 How to plan and work effectively

- **Develop a self-management system that works for you** – review its success at frequent intervals and modify, as appropriate.
- **Learn from your experience** – understand what has been successful in your approach and why, then make appropriate changes to aspects that you find less useful.
- **Learn from others** – discuss with friends and fellow students to see what works for them. Swap tips with each other – you may pick up some useful ideas to try out.
- **Set yourself realistic aims and objectives** – better to complete a smaller task than feel that a major undertaking is beyond your reach, leading to frustration.
- **Avoid procrastination** – find a way to get started; if you don't, the work levels required will increase, making everything more difficult.
- **Avoid distractions** – switch off your phone/email notifications so that you can work without 'e-disturbance'. Similarly, avoid physical distractions, such as the TV, etc.
- **Try not to be a perfectionist** – perfection is wasteful of effort.
- **'Nudge' yourself into better habits and successful routines** – the idea behind behavioural nudging is that minor actions can make it easier for you to make a desired choice. Applying this to yourself, you might, for example, leave your phone in a separate room, so you avoid being distracted by incoming messages when studying, or you might arrange to meet a friend near to the library, so you have an easier route to go there afterwards.
- **Try to concentrate effectively** – a series of short, focussed spells at working can achieve much more than a long spell of aimless, vague study.
- **Avoid recurring crises** – if you keep getting into the same time-management issues, identify and solve the underlying problem.
- **Learn to say 'no' firmly** – suggest an appropriate time for social activities, so they don't interfere with your schedule.
- **Know your own body rhythms** – decide whether you are a morning, afternoon or evening person, and focus your academic efforts during your active time(s).
- **Learn to recognise the benefits of rest, relaxation, exercise and sleep** – and build all of these elements into your timetable.
- **Take short but complete breaks from your tasks every 40–60 minutes** – you will return to them refreshed and ready to work. Set your smartphone's alarm so that you don't forget.
- **Work in a suitable study area** – keep your workspace organised.
- **Avoid clutter** – both in your study environment and in your mind.
- **Learn how you learn best** – see Chapter 3 for advice on how to learn and how to remember information.
- **Learn to access and use information effectively** – follow the tips in Chapter 4.
- **Learn to take notes effectively** – follow the tips in Chapter 3.
- **Write efficiently** – use word processor features to speed up your use of time (Chapter 14).

Achieving quality in time management – avoid wasting time in unproductive studying, e.g. reading a textbook without having specific objectives for that reading (see p. 28–30 for advice on 'active reading' and Chapter 3 for advice on effective learning).

Being assertive – often friends and family can spoil your plans by interrupting you. Find a way of preventing their interruptions; for example, put a 'do not disturb' sign on your door, or work somewhere where you won't be disturbed.

Avoiding time-wasting activities

Ineffective or wasted time is that spent doing activities that produce no benefit in relation to your aims. This doesn't necessarily mean that all discretionary time is wasted – we all need rest and relaxation in appropriate amounts. The real time-wasters include: procrastination; chatting; over-long breaks; uninvited interruptions by others; and even ineffective study. If any of these are a problem, then you need to find strategies to minimise their effect, such as studying in a library, or setting limits to the time allocated to meet with friends. As an example, if you find yourself spending too much time using social media, try to limit this to a specific period each day. You might even use this activity as a 'reward' when you achieve a certain milestone.

It is also important to match your activity to your body's rhythm. Everyone has times of day when they feel more alert and more able to work or study. Work out when these times are for you and plan your work accordingly. Allocate relaxation events for periods when you tend to be less alert. Make sure that you get enough relaxation and sleep – research shows that we all need rest and sleep of sufficient duration and quality to process each day's information (p. 19).

Reviewing your progress

It's not enough just to create a set of objectives. You must revisit them to check on progress and update them when necessary. This process of review applies to the simplest of daily checklists, but also to your longer term aims. Accomplishing your milestones and objectives gives a feeling of achievement – but equally, not completing others might provide a prompt to start working in a more focussed way.

Managing your well-being

There are many aspects of well-being, from fitness to mental contentment. As it applies to academic work, the chief issues include: motivation, physical health, anxiety and stress. In relation to general student life, key issues include: relationships, financial issues and mental health. Box 2.2 provides some practical suggestions related to these matters.

Some well-being issues are transient, such as the stress experienced before an exam or oral presentation; others may be deep-seated and longer lasting, such as a relationship break-up. Each issue affects us individually, drawing on our personality, experiences and medical history. It is therefore impossible to provide specific advice – you must draw what you can from general guidance, as it applies to your situation.

KEY POINT If you are worried about any well-being issue, it is vital to seek professional help as soon as difficulties materialise. For academic matters, speak to your personal tutor or course leader; for health conditions, ask your doctor or your university's health service; and for relationship and mental health issues, talk to your university's student welfare staff.

Text reference

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Box 2.2 How to approach well-being issues

- **Staying motivated** Most students enjoy their course and the opportunity to study their chosen subject in depth. However, you may experience ups and downs in your motivation. These might be related to changes in subjects/topic, a response to a less-than-inspiring lecturer, or a low mark in an assessment. Usually these changes are temporary: should you feel unenthusiastic at present, you will soon be more upbeat again. Sometimes, however, your feelings may be more deep-rooted and worrying – possibly you feel either unsuited to university as a whole, or that you may be on the wrong course. If this applies to you, contact your course leader, academic tutor or study adviser: there are often pathways to change subject within institutions. Of course, this assumes that there are places available and it may involve extra work in catching up on coursework. Note that if you don't make a change within a certain period, you may be 'timed out' of the process. The sooner you raise the issue, the better.
- **Being healthy** One of the keys to mental well-being is physical fitness and lack of illness ('a healthy mind in a healthy body'). All universities have gym and sports facilities and also a range of sports clubs and societies, so there are many opportunities to take part in some form of physical activity. Diet is another important aspect of physical well-being, and if you are feeling sluggish and generally run down, maybe you should review your eating and drinking habits. Take steps to adjust appropriately. Leading a hectic life could be an additional issue. Are you getting enough relaxation and sleep? Ongoing medical conditions can also have an effect on your mental state. It is important to tackle these issues as early as possible. For example, if you find that conditions such as migraine, or chronic joint or muscle pain are affecting your ability to study, consult your general practitioner or the university doctor.
- **Dealing with anxiety and stress** While anxiety is a feeling of worry or uneasiness about situations with uncertain outcomes, stress is a state of mental tension arising from your perception of adverse or challenging circumstances. Anxiety can result from being in an unfamiliar environment and interacting with new people, while study workload is a recognised cause of stress among students. This factor is heightened near to assessment deadlines and exam time. Being well organised in your studies and revision can help to reduce anxiety, but a certain amount of nervousness is inevitable and may even be beneficial. Recognise that such feelings are a natural way of boosting your performance, and are important for motivating and energising you. A common cause of anxiety is 'imposter syndrome', a feeling that you are not worthy of your place at university – either because you doubt your ability, or you lack the confidence to proceed with self-directed study at this level. Be assured that others believe in you: the education authorities who gave you the qualifications that got you to university, the teachers who supported you, the university recruitment staff and your friends and family members who will back you up. Try to battle through any feelings of low self-worth: it won't be long before your positive personal qualities will see you through and/or you realise that your fellow students and tutors are not as intimidating as they may at first appear.
- **Working through relationship issues** Being homesick is probably the first emotional response of any student living away from home, while a feeling of loneliness affects many first year students, even if they live at home. Existing personal relationships will inevitably change. The resulting adjustments can cause anxiety. These negative feelings can be conquered by a determination to engage with others, for example, in clubs, societies and in your academic classes. Maintaining contact with your family and old friends provides a valuable anchor to help you work through these feelings, but it is important to realise that making new friends and dealing with new situations are skills that you will require throughout your working life. General advice about difficulties in relationships can appear trite and simplistic – such as 'talk things through'. However, such advice incorporates more than a grain of truth. Sometimes a period of worry and sadness is inevitable, e.g. after a break-up or the illness of a close family member – it helps to understand that time will lessen your negative feelings.
- **Overcoming money issues and debt** Financial issues can be a problem for many students, with the timing of loans causing times where you have plenty of money, followed by periods where money is in short supply. The strong advice here is to create a budget for each semester and do your best to stick to it (for specific advice on budget-setting, see McMillan and Weyers, 2021). For some undergraduates, having a large debt like a student loan is a major cause of worry. If so, talk to those managing your account at your bank. They will be used to dealing with this sort of issue and can offer helpful advice and support, for example, through overdraft facilities. Your student union or university advice service might also have a specialist officer who can give advice and support.

STUDY EXERCISES

- 2.1 Evaluate your time usage.** Compile a spreadsheet to keep a record of your daily activities in 15-minute segments for a week. Analyse this graphically and identify areas for improvement.
- 2.2 List your short-, medium- and long-term tasks and allocate priorities.** Produce several lists, one for each of the three timescales and prioritise each item. Use this list to plan your time management by scheduling high-priority tasks and leave low-priority activities to 'fill in' the spare time that you may identify. This task should be done on a regular (monthly) basis to allow for changing situations.
- 2.3 Plan an 'elephant' task.** Spend some time planning how to carry out a large or difficult task (learning a language or learning to use a complex computer program) by breaking it down into achievable segments ('bites').
- 2.4 Research the support services at your university.** Some are run by the university itself, others in tandem with the students' association. Where are they physically situated and what Web resources do they have? Don't ignore the 'help desks' associated with the library and IT services, which can provide valuable practical support.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

3 Learning effectively

Understanding the learning process – it is important to focus not only on the *products* of learning, but also on the *process* of learning, as this will enhance your capacity to learn.

The ability to learn is such a fundamental human characteristic that it is often taken for granted. However, like any skill, learning is something that can be improved by reflection, focus and effort. This chapter aims to provide you with information and practical advice to help you consider your own practices and preferences, so that you can improve your approach to study and enhance your understanding of biology at a deeper level.

KEY POINT At university, you will be expected to set your own agenda for learning, as a key aspect of self-management (Chapter 2). While there will be timetabled activities, assessments and deadlines, it will be your responsibility to decide how best to study and learn, how to manage time and tasks and, consequently, what you will gain from the experience. You should be willing to challenge yourself, to make the most of your time and to realise your full potential.

Thinking about thinking: metacognition

The cognitive processes that you will be expected to use in your learning are often described as a sequence, starting with so-called ‘shallower’ thought processes and ending with ‘deeper’ processes, each of which builds on the previous level, as shown in Table 3.1. The first two categories in this ladder apply to gaining basic knowledge and understanding (facts and information), relying strongly on memorisation; these are important when you first encounter a new topic in biology. Categories 3 to 6 require you to apply, analyse, evaluate or create something, going beyond simple knowledge; these higher-level processes are especially relevant to the later years of study, and to researchers and professional scientists. Sometimes the term ‘critical thinking’ may be used to describe them collectively. Your tutors will want to reward the deepest thinking appropriate for your level of study – this is often signified by the words they use in assessment tasks and marking criteria (column four, Table 3.1 and p. 64). While the link between these levels and assessment criteria is not an exact one, being aware of this relationship from the outset can help you to gain more from your studies, since you will understand better what is being asked of you.

Definitions

Cognition – the process of thinking; acquiring and using knowledge and understanding.

Metacognition – understanding how you think and how you apply thinking processes to your learning.

Definition

Critical thinking – this term is often used to cover a combination of some of the higher levels of thinking, such as analysis, synthesis and judgement. This type of thinking is logical, cutting through complexity, using evidence to develop a clear position on a particular issue. It is particularly applied to the analysis of a problem and the presentation of a solution to it.

Understanding the learning process

In essence, to *learn* something is to be able to commit it to long-term memory, to be able to recall it and, through understanding, apply it when needed; this is true of something as simple as the assimilation of factual information in first-year classes, through to complex processes such as the application of the scientific method during a research project (Chapters 29 and 32). The human brain processes far more short-term information than it is able to store in long-term memory. Additionally, long-term memories are not fixed forever – the brain processes and reprocesses information, deleting some memories, amending others and laying down new ones. To maximise your learning, you need to be aware of what approach works best for you to receive and understand information so that it is retained in your long-term

memory. Box 3.1 summarises some of the key outcomes of research into learning, highlighting how you might apply these findings in your studies.

KEY POINT It is important to realise that it is the *process* of analysing your own thinking and learning (metacognition) that is critical to increasing its effectiveness. The best way to analyse your learning is through self-evaluation (testing your ability to retain and use information).

Table 3.1 A sequence of cognitive processes, moving from 'shallower' thought processes (top of table) to 'deeper' levels of thinking (bottom of table). This table is derived from research by Benjamin Bloom and colleagues (Bloom *et al.*, 1956; Anderson *et al.*, 2001). When considering the cue words in typical question instructions, bear in mind that the precise meaning will always depend on the context. For example, while 'describe' is often associated with relatively simple processes of recall, an instruction like 'describe how the human brain works' demands that you exhibit higher-level understanding. Note also that while a 'cue word' is often given at the start of a question/instruction, this is not universally so

Thinking processes and description (in approximate order of increasing 'depth')	Example in life sciences	Example of typical question structure, with cue word highlighted	Other cue words used in question instructions
1. Remembering (knowing facts). If you know information, you can <i>recall</i> or <i>recognise</i> it. This does not always mean you understand it at a higher level.	You might know the order of bases in a piece of DNA but not understand what this means.	<i>Describe</i> the main components of a biological membrane.	<ul style="list-style-type: none"> define list state identify
2. Understanding. To understand information means you can <i>construct meaning</i> from it.	You might know the order of bases in a piece of DNA and understand that they code in triplets for specific amino acids.	<i>Explain</i> how membrane components are involved in the accumulation of solutes within living cells.	<ul style="list-style-type: none"> contrast compare distinguish interpret
3. Applying. To apply a fact means that you can <i>put it to use</i> in a particular context.	You might be able to take the DNA base sequence and work out the amino acid sequence of the protein for which they code.	Using the Nernst equation, and realistic values for the membrane potential and solute concentrations, <i>demonstrate</i> how Na^+ ions must be actively transported out of the cells of marine organisms.	<ul style="list-style-type: none"> calculate illustrate solve show
4. Analysing. To analyse information means that you are able to <i>break it down into parts</i> and show how these components <i>fit together</i> .	You might be able to construct a three-dimensional model of a protein derived from the base sequence.	Drawing on information about membrane structure, <i>defend</i> the endosymbiotic theory of eukaryote evolution.	<ul style="list-style-type: none"> compare explain consider infer
5. Evaluating. If you evaluate information, you <i>arrive at a judgement</i> based on its importance relative to the topic being addressed.	You might be able to comment on theories about how a protein has evolved, by considering the structure of related proteins and relating this to the taxonomic position of their source species.	<i>Evaluate</i> the relative importance of passive and active transport in the accumulation of heavy metal salts by the main groups of soil fungi.	<ul style="list-style-type: none"> review assess consider justify
6. Creating. To create or synthesise ideas, you need to be able to <i>extract relevant facts</i> from a body of knowledge and use these to <i>address an issue in a novel way</i> or <i>create something new</i> .	You might be able to work out the function of a protein for which you know the sequence of bases, based on a comparison with other like proteins.	<i>Devise</i> an experiment to test the hypothesis that a specific membrane fraction contains a functional ATPase involved in glucose transport.	<ul style="list-style-type: none"> design integrate test create

Being adaptable in your learning – you should be prepared to adjust your study methods according to the topic, the learning environment and the approach of your lecturers (Box 3.2).

Being active in your learning – a common theme in all recommended approaches to effective learning is *activity* on the learner's part. Thus, active note-making, or marking up a *PowerPoint* handout with your own hand-written comments and annotations during lectures, requires a greater level of engagement with the material than the more passive action of simply listening, or looking at your laptop screen. Studying with your peers, for example, by discussing concepts or debating issues with other students, involves a deeper degree of thinking than the surface thought processes involved in reading text. In both cases, you are likely to learn (understand and retain) more, as discussed in Box 6.3.

Your preferred approach to learning is simply the one that suits you best – for example, many students describe themselves as ‘visual learners’, making the most of diagrams and images. This fits with cognitive research that shows vision to be the dominant sense for most people. Others have their own preferences, such as summarising written material as bullet-point lists of ‘headline’ facts and concepts. For your part, you will need to be flexible in your approach, since different tasks will require different strategies. For example, many of the outputs assessed at university, such as exam answers, essays and reports, involve individual study and carefully crafted written answers, whereas practical skills are best learned through ‘hands-on’ activities – learning by doing.

Learning effectively in different settings

While how you learn best depends on your personal characteristics, your approach may need to be modified to suit the different modes of teaching you encounter.

Learning from lectures

These are designed to impart knowledge and understanding efficiently on the part of the staff. The best lectures can stimulate interest and motivation, helping you to appreciate the ‘big picture’. They can also help to correct misunderstandings, for example, in question-and-answer sessions during or after the lecture. The most effective lectures go beyond dictation and fact-telling, challenging the audience to think more deeply about a particular topic. Therefore, passive listening to live presentations or recorded lectures and videos without taking notes is an ineffective approach to learning – you need to *actively engage* with the session by making your own notes as you watch and listen, forcing you to translate your understanding into written form and, thereby, beginning the process of remembering the material (for more details on note-making, see Chapter 4). To optimise your learning from lectures, you need to:

- **Prepare beforehand** by finding out what the topic will be, what the learning outcomes are, and doing some preliminary reading, and even some self-assessment (pretesting, Box 3.1).
- **Arrive in good time**, sitting where you can see and hear the lecturer, with the right materials for taking notes.
- **Listen attentively**, particularly when the lecturer is stating what he or she plans to cover; when they indicate that you are expected to write things down word-for-word; when a definition is being presented; when key facts are stated (often emphasised by repetition); and especially at the beginning, when key aspects may be emphasised, and at the end, when the take-home messages are usually covered.
- **Create useful notes** – these should *not* be a simple transcription of the lecturer's words, but rather your personal summary of the key points that have been made (see Chapter 4). This is especially so where the material is provided to you as a *PowerPoint* or *Word* file (or equivalent) – you should optimise your learning by explaining and interpreting the material for yourself, thereby laying the foundations for remembering the content. Note that tactics such as highlighting and underlining of printed text have been shown to be ineffective as aids to learning – you need to actively engage with lecture material in whatever ways work best for you.

Box 3.1 How to improve your memory and learning

Consider the following ideas:

- **To learn effectively, you need to transfer information from short-term (working) memory to long-term memory.** Studies show that this is best achieved in short, focussed sessions in an environment that is free from distractions (no multitasking!). Since we can maintain maximum focus for up to 20 minutes at a time, you may find it helpful to take several breaks for every hour of study. Self-assessment (see below) is one way of breaking up your learning periods into small blocks of time, with the added bonus that it will enhance your learning.
- **Spacing out your learning is more effective than cramming into a single block of time.** The ‘spacing effect’ describes the finding that more information is encoded into long-term memory when study is spaced out across several days, or longer. This has applications for scheduling your revision (p. 109) and other study periods; little and often is better than all-together. Rest and relaxation also helps to space out your study, enhancing your long-term memory and recall.
- **Retrieval and reprocessing is essential to retaining information in your long-term memory.** Hermann Ebbinghaus described the ‘forgetting curve’ in the late nineteenth century, showing that most of the information that is learned on a particular day is forgotten within the next day or so, decaying with a half-life of around 24 hours. Subsequent studies have shown that spaced retrieval (remembering) and review enables the information to be reprocessed and retained for longer, enhancing the consolidation of long-term memory (see Fig. 3.1). As a practical example, this means that you will learn more from retrieving and reviewing material a day or so after first reading than you will from passively re-reading written text. It is perhaps best summed up by the maxim: ‘repeat to remember – remember to repeat’.
- **Exercise well to think well.** Exercising will raise your heart rate, boosting the blood flow to all parts of your body, including your brain. Exercising increases alertness and attention and has a positive effect on mental health, enhancing motivation and reducing anxiety. Aim to schedule regular breaks – even a few minutes of exercise between study sessions can help you learn more effectively.
- **Sleep well to think well.** Sleep is necessary to process and encode your long-term memories. Conversely, lack of sleep impairs your attention, working memory, higher skills such as reasoning, and your physical dexterity. While you might occasionally work an ‘all-nighter’ to make a deadline, you would be better to plan ahead and schedule your time to make the most of your sleep.

- **Self-assessment enhances learning.** This is true whether you use an informal approach – for example, stopping after every section of a written article and trying to recall and jot down the main points – or a more formal approach, such as answering questions from past exam papers. You can also apply self-assessment *before* a period of learning (‘pretesting’). For example, before a lecture on a particular topic you might try to write down as much as you know about the subject. Doing this will ‘prime’ you to retain more of the novel information delivered in the lecture and may also highlight some aspects where you have misconceptions, enabling you to correct these in your long-term memory.
- **Teaching a subject can be a useful approach.** In order to explain something to someone else, you need to fully understand it yourself. For example, you could work with a ‘study buddy’, explaining concepts and ideas to each other – learning with and from your peers can be a valuable approach. Even without a partner, you can simply speak your explanations out loud – either to yourself, to your phone’s audio recorder or even to your family pet!
- **Apply a scientific approach to your own learning,** based on observation of what works for you and experimentation using different approaches, with evaluation based on self-testing and assessment results.

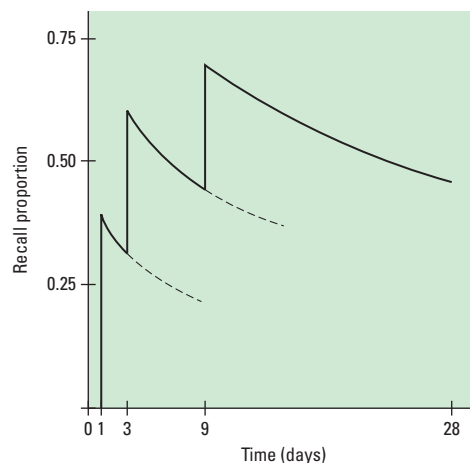


Fig. 3.1 A representative ‘forgetting and retrieval’ curve for memorisation, based on Kang *et al.* (2014). This graph shows the impact of spaced retrieval at 1, 3 and 9 days on subsequent recall. Note that the curve becomes less shallow, implying slower forgetting, with each iterative retrieval leading to enhanced retention in long-term memory.

Definition

Take-home message(s) – the main point or points that a presenter wishes you to remember from what they have said (perhaps a summary of facts or a set of conclusions) and usually emphasised at the end of a lecture, for example. If a lecture or seminar has been particularly detailed or complex, noting these messages will help you to make sense of it on revisiting/ revising – and they may be closely related to what is eventually examined.

Laying the foundations for learning – unless you are gifted with photographic recall, it is not easy to commit information to your memory unless you have a conceptual framework of understanding in which to 'place' it. This 'bigger picture' comes from reading, listening and thinking at an early stage in the process, by asking questions and discussing with others.

- **Ask questions if you do not understand something.** This might not be during the lecture, because uncertainties or misunderstandings may become apparent only when you go over your notes, read a textbook, or talk about the topic with a fellow student. However, it should be as soon as possible after your question comes to mind, with opportunities to enquire at tutorials, via email and online discussion boards, or at later lectures or meetings. Most lecturers welcome the opportunity to answer questions, as it enables them to correct misconceptions arising from their presentation.
- **Above all, you *should* attend lectures,** even if you feel their value may be limited in some cases – do not underestimate the way that looking, listening and thinking contribute to understanding and learning. Also, facts on the page rarely have the additional context or emphasis that a good lecture can provide.

Box 3.2 provides some tips for dealing with some of the different teaching styles of lecturers.

Learning from laboratory classes

Practical work is an essential component of biology education, so that your understanding is grounded in real examples of specimens, equipment, processes and reactions. Within your degree programme, laboratory sessions will be used to stimulate your interest and support your learning, fulfilling a range of objectives, including:

- **applying relevant health and safety protocols** (Chapter 20), including the use of appropriate personal protective equipment (Chapter 19)
- **illustrating concepts and principles introduced in lectures and other taught classes**
- **allowing you to encounter at close hand examples of organisms, materials and equipment that will enhance your learning**

Box 3.2 How to accommodate the teaching styles of different lecturers

One challenging aspect of lectures is that no two staff members deliver them in the same way. This means you will need to adjust your listening and note-making approaches to suit. For example:

- **It may be hard to extract facts and meaning** from a lecturer who tells meandering stories or keeps the class amused with anecdotes. You must not lose focus when listening and you need to be alert when the key points are mentioned; use your textbooks to fill in any missing material when making up your notes later on.
- **You may be distracted** if the lecturer uses technology in a way that makes you think about the medium rather than the message. By all means enjoy the videos, images and electronic 'bells and whistles', but do remember to listen out for the underlying messages and focus your notes on these.
- **It may be difficult to stay attentive** if the lecturer's delivery is quiet, monotonous or mumbled (or all three). Here, you will need to ensure that you sit where you can hear, and listen very carefully for key words and phrases, rather than changes in intonation, that might signal importance, or a change of emphasis. Do not be afraid to ask the lecturer to speak up if you cannot hear – most will welcome this feedback.
- **You may find it difficult to abstract general principles** if the lecturer provides great detail about a particular 'pet' topic or current research work. Moreover, the latter may be hard to follow if complex jargon and undefined acronyms are used. The remedy here may be to use the textbook before and after the delivery to create an overarching framework of understanding.

Learning by making use of all of your senses – while the main sense you will use in practicals and field excursions will be sight, on occasion other senses will be important, such as hearing, smelling and touch. Taste is unlikely to be used, for obvious health and safety reasons.

- **developing manipulative skills in relevant laboratory techniques**, as detailed throughout this book
- **applying the principles of scientific method and experimental design**, Chapter 29
- **learning the skills involved in data collection, manipulation and analysis** (Chapters 27, 71 and 72), including mathematical problem-solving (Chapter 75) and statistical analysis (Chapters 76 and 77)
- **evaluating the validity and reliability of observations and results, including possible sources of error**
- **developing abilities in data presentation using tables (Chapter 73) and graphs (Chapter 74)**
- **encouraging active and collaborative learning (Chapter 3)**
- **learning how to design and manage a scientific project (Chapters 29 and 32).**

You will maximise your learning in practical sessions by thorough preparation, as detailed in Chapter 19.

Succeeding in problem-based learning (PBL)

In this teaching method, you are presented with a ‘real-world’ problem or issue, often working within a team (Chapter 7). As you tackle the problem, you are expected to gain factual knowledge, develop skills and exercise critical thinking (Chapter 6). Because there is a direct and relevant context for your work, and because you have to employ active learning techniques, the knowledge and skills you gain are likely to be more readily remembered and then applied to other scenarios in the future. This approach also more closely mimics workplace practices. PBL usually proceeds as follows:

1. **You are presented with a problem** (for example, a case study, a hypothetical scenario, a topical issue).
2. **You consider what issues and topics you need to research**, by discussion with others if necessary. You may need to identify where relevant resources can be found (Chapter 5).
3. **You then rank the issues and topics in importance**, allocating tasks to group members, if appropriate.
4. **You carry out the necessary research**, for example, using methods described in Chapters 4 and 5
5. **You review the information that has been obtained**. As a result of your research, new issues may need to be explored and, where appropriate, allocated to group members for further study.
6. **You produce the requested outcome, such as a report, diagnosis, seminar presentation or poster**. An outline structure will be required, and for groups, further allocation of tasks to accomplish this end-point.

If asked to carry out PBL as part of your course, it is important to get off to a good start. At first, the problem may seem unfamiliar. However, once you become involved in the work, you will quickly gain confidence. If working as part of a group, make sure that your group meets as early as possible, that you attend all sessions and that you do the necessary background reading. When working in a team, a degree of self-awareness

is necessary regarding your ‘natural’ role in group situations (Chapter 7). Various methods are used for grading PBL, and this may involve peer assessment (Chapter 9).

Learning from tutorials

These may fulfil a number of purposes, including:

- **providing guidance**, as part of an advising system
- **reviewing and testing your understanding** of material covered in lectures and lab classes
- **introducing new material** that is better taught in small groups
- **developing and assessing writing, numerical and problem-solving skills through appropriate tasks**
- **promoting interactions among students and with the tutor.**

The face-to-face discussions that can occur during tutorials are rarely possible in larger classes and they add value by enabling you to develop your views and understanding through an exchange of opinion and knowledge. Think of your tutor as a potential ally. For example, he or she may be able to explain things differently from the lecturer, thereby helping you to understand any concepts that you find difficult. Never feel embarrassed about asking questions – the tutor is there to help you learn and the chances are that if you find a subject tricky, so will the rest of the group. Your tutor may also have responsibilities beyond teaching, and may be able to help you with subject choices or non-academic issues.

To get the most from tutorials, you will need to prepare beforehand, for example, by:

- **carrying out the recommended reading, or tackling the set problems**
- **submitting expected work in good time**
- **thinking about the issues involved in the subject matter**
- **preparing any questions you might have about the topic you have been asked to study**
- **reflecting on the potential relevance of the tutorial to your module and course.**

Learning online and in blended models

Most courses now include elements of online learning (e-learning) within the curriculum, with distance learning programmes relying almost entirely on this mode of delivery. While online learning allows easy access to learning materials at a time and place of your choosing, it also assumes a level of personal discipline in your approach to study that requires organisation and effort on your part. Effective learning in this context requires the following:

- **making regular and frequent visits to each module in your university’s online learning portal** – log in every couple of days to your virtual learning environment (VLE), for example, Moodle, Sakai, Blackboard
- **allocating time to study** – schedule a specific time and day for online learning sessions within your weekly timetable
- **paying special attention to announcements, messages and emails** – where appropriate, add these to your smartphone calendar
- **participating in online discussions** – this can be particularly useful for students who are studying in distance mode, providing them with opportunities to engage with others

Taking account of tutorial assessment – the tasks prepared for tutorials may be marked and in some cases your participation will be graded. Consult the course handbook to find out what proportion of the total mark derives from tutorials and what the marking criteria are. Ensure that you maximise the marks obtained from this source, as they may be relatively easy to achieve if you put in the necessary work.

Communicating online – email and discussion forums require appropriate ‘etiquette’. Always be polite, friendly without being over-familiar, and take care not to be abrupt or confrontational. If in doubt, do not send your message right away; reread later and consider how others might view what you say. A useful approach is only to send what you would be happy to hear being read out aloud to classmates.

- **organising learning materials in digital form** – for example, by creating lists of ‘live’ links to website addresses
- **reading the advised materials** – online or as printed texts
- **make your own notes from online lectures and learning materials** – simply downloading a file to your computer is not equivalent to actively engaging with the content of the file
- **using active learning techniques** – rather than passively reading and re-reading text (Chapter 9)
- **observing staff-set timelines in study, assessment and submission of work**
- **creating personal milestones** – in study, self-assessment and preparation of work.

Box 3.3 gives further advice on strategies to maximise online learning.

Box 3.3 How to get to grips with online learning (e-learning)

Some key aspects of tackling online learning are outlined below.

1. **Develop your IT skills, if required.** While online learning requires only basic keyboard and Web skills, if you feel that these need strengthening, you should attend courses offered by your university.
 2. **Develop a routine and timetable for online learning.** This should include regular engagement with learning materials in the VLE, and also a timeline for each assessment. Staff will often update information and present changes through the ‘announcements’ section of the VLE. They may post information about assessments and links for assessment submission. Most portals also have opportunities for peer-to-peer discussion.
 3. **Make the most of online lectures and video presentations.** Since you are free to choose when and where to watch, you can optimise your learning by viewing at a time and in a location when you learn best. You can also replay a section that you found difficult to decipher or understand.
 4. **Participate.** Effective online learning requires you to take an active approach.
 - At the start of each new course, spend some time getting to know what is available online to support your learning. As well as learning resources, this may include crucial information, including learning outcomes (p. 64), dates of submission for coursework and assessment criteria.
 - If you are able to download lecture notes (e.g. in the form of *PowerPoint* presentations), do not think that simply reading through these will be an adequate substitute for attending lectures and making further notes – you will need to use active learning techniques such as annotation (p. 18), recall and self-evaluation (Box 3.1) to maximise your learning.
 5. **Organise your files and Web links.** Take the time to create a meaningful system of folders and files for downloaded material in tandem with your own coursework files and set up folders on your Web browser for bookmarked websites. *Always remember that you need to make at least one back-up copy of each important file, storing this well away from your working copy – ensure that the same accident cannot happen to both copies.*
 6. **Take special care when submitting your coursework.** Always check that you are sending the correct, up-to-date version and make sure you keep a back-up of any file you email or submit online. Follow instructions carefully, for example, regarding file type or how to use your portal’s ‘digital drop-box’.
- Get involved in discussion forums: ask questions; start new threads; answer points raised by others, if you can.
 - Try to gain as much as you can from formative online assessments (p. 64). If these include feedback on your answers, make sure you learn from this and consult your tutors if you do not understand it.
 - Learn from any descriptions that your lecturers provide of linked websites. These pointers may help you to evaluate such resources for yourself in the future (pp. 44–46).
 - Help your lecturers by providing constructive feedback when they ask for it. You may find this easier to do online, rather than hurriedly filling out a feedback sheet at the end of a face-to-face class.

Blended learning makes use of on-campus education – either in weekly sessions, or with block attendance – and online learning. While blended learning may provide greater control over the path and pace of some aspects of your learning, it will often require you to follow a particular sequence of face-to-face and online components, requiring organisation and self-discipline on your part. As an example, the so-called ‘flipped classroom’ model requires students to complete their ‘homework’ – typically watching video lectures or reading relevant text – in advance of face-to-face classes, which are then devoted to group-based collaboration, including problem solving, question-and-answer sessions and other peer-to-peer learning activities aimed at higher cognitive levels, such as analysis, evaluation and synthesis (Table 3.1). The flipped classroom can also provide enhanced opportunities to engage with feedback during on-campus teaching sessions.

Reflecting on your learning

Reflection can be described as the process where an individual evaluates an experience to arrive at a deeper understanding of the incident(s) and surrounding issues. The process is especially valuable as you make the transition from your past learning experiences to university learning, with its emphasis on self-guided study and its different assessment methods (Chapter 9). In particular, coming to terms with the higher levels of thinking required at university (Box 3.1) means that you may need to alter the approaches that you have taken in the past. Be prepared to change your learning methods if you find that they are unsuccessful or difficult to apply in particular circumstances.

KEY POINT The only person who can judge the effectiveness of your learning at university is you. Only you will know how much effort you put into your studies and what you are therefore expecting, in terms of a mark or grade; only you will know how comfortable you feel with a particular approach to learning; and only you can respond to the feedback you have been given, in terms of how you should learn for future assessments.

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STUDY EXERCISES

3.1 Review your approach to learning. Find a website that lists some tips for improving your learning (for example: <https://www.lonsdaleinstitute.edu.au/blog/10-scientific-study-tips-improve-learning/>). Read through the list and identify those that you already practise, plus one or two to try out for yourself. Use self-assessment (Box 3.1) to see if they work for you and incorporate any successful examples into your learning strategies.

3.2 Review your note-making methods. How well suited are they to your needs? How well suited are they to the lecture styles of your academic staff? Have you captured the important points, or are you merely transcribing

exactly what the lecturer says? Where lecturers use *PowerPoint* slides, are you listening for, and capturing in note form, the additional spoken points and examples that the lecturer is adding during the live presentation?

3.3 Think about your thinking. Read through Table 3.1 and consider different thinking processes in relation to (a) feedback you have received from a previous assignment or (b) your next assignment and your intended approach to it. Does this reveal that your marks could be improved by thinking more deeply about the topic, or by capturing your thoughts more clearly in your writing?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

4 Making the most of learning resources

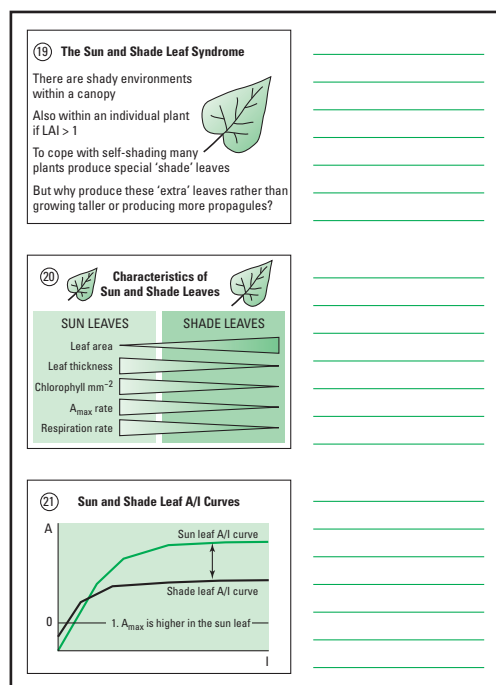


Fig. 4.1 An example of a printout from PowerPoint in 'Handouts (3 slides per page)' format, showing handwritten annotations (note-making).

Choosing note-making methods appropriately – the method you choose to make notes might depend on the subject; the lecturer and their style of delivery; and your own preferences.

Comparing lecture notes with a classmate – looking at your notes for the same lecture may reveal differences in approach, depth and detail that may prove mutually beneficial.

You are likely to use a range of learning resources during your time at university. These include: lectures and presentations – either face-to-face or online; printed materials – including books and scientific papers; and Internet resources – either contained within your university's virtual learning environment or within the broader Internet. This chapter aims to provide you with strategies to optimise your learning for each resource type.

Making the most of lectures and presentations

Creating your own notes is the most effective way to learn from lectures and presentations (Chapter 3). This is especially true in sessions where the handouts of presentation slides are made available (for example, *PowerPoint* slides). Simply reading through a handout as the lecture progresses is a superficial and ineffective approach, resulting in very little learning – also, if the slides mainly consist of headings or images, you may miss some of the key points being made. Tactics such as highlighting and underlining of printed text have been shown to be ineffective as approaches to learning – you need to actively engage with lecture material in whatever ways work best for you. For example, annotating the material with your own thoughts and ideas – either in handwritten form for printed copies, or on your laptop for digital files – leads to greater understanding and retention of material. However, a 'slides handout' can free you from the need to copy out large diagrams, and the basic text and images can provide a structure for the lecture that you can easily add to with your own notes. If you are not supplied with such handouts in advance, you may be able to print out the presentation beforehand, perhaps in the '3 slides per page' format that allows space for notes alongside each slide (Fig. 4.1). Make time to scan through this before the lecture; then, during the presentation, focus on listening to what the lecturer has to say. Note down any extra details, points of emphasis and examples. After lectures, you can also add notes from supplementary reading, where appropriate.

KEY POINT Good performance in assignments and exams is built on effective learning and revision (Chapters 9, 17 and 18). However, both ultimately depend on the quality of the notes taken from lectures, texts and Internet resources.

Making legible and meaningful lecture notes is essential if you are to make sense of them later. For handwritten notes, begin by noting the date, course, topic and lecturer on the first page of each day's notes or handout. Number every page in case they get mixed up later. The most popular way of taking notes is to write in a linear sequence down the page, emphasising the underlying structure via outline headings, as in Fig. 11.3. However, the 'pattern' and 'Mind Map' methods (Figs 4.2 and 4.3) have their advocates: experiment, to see which method works best for you.

Making lecture notes with a laptop or tablet frees you from any issues with illegible handwriting. In addition, you are likely to be able to type faster than you can write, helping you to keep up-to-speed during a fast-paced

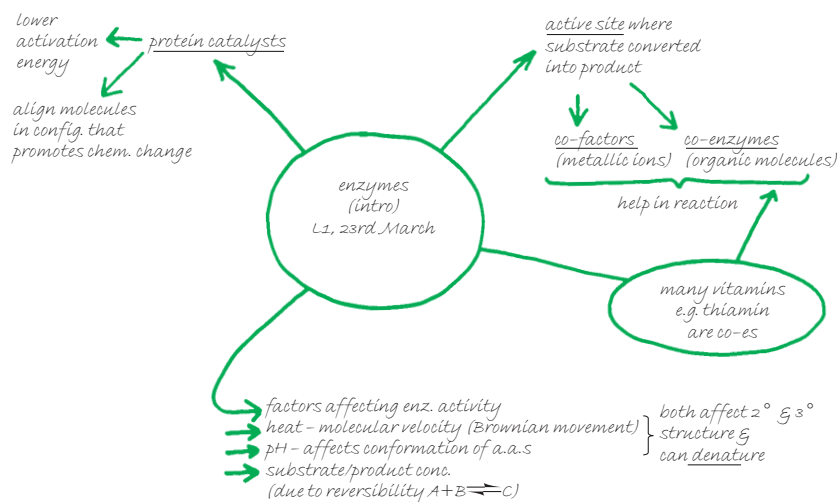


Fig. 4.2 An example of 'pattern' notes, an alternative to the more commonly used 'linear' format. Note the similarity to the 'spider diagram' method of brainstorming ideas (Fig. 10.2).

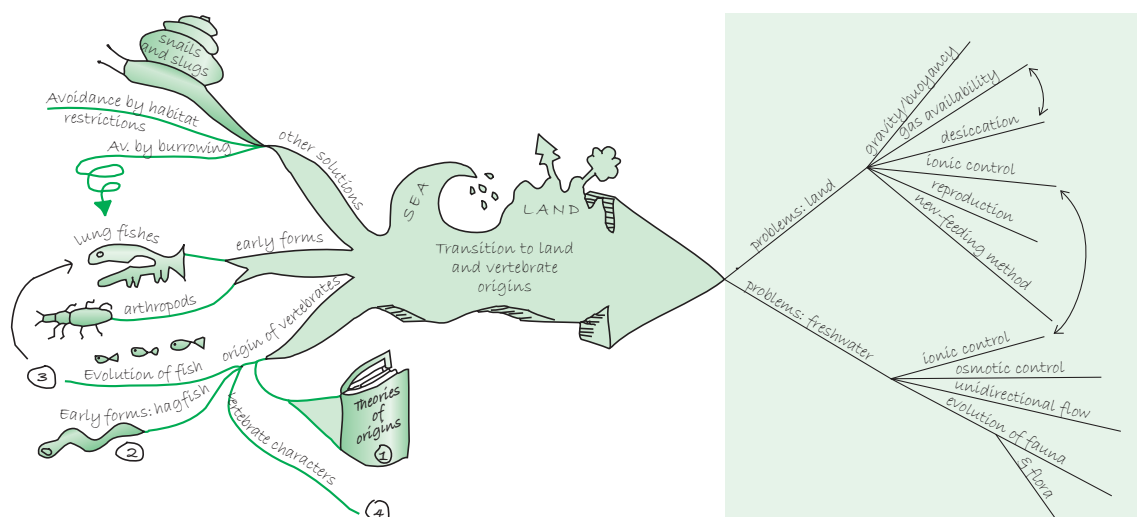


Fig. 4.3 Example of the 'Mind Map' approach to notetaking and 'brainstorming'. Start at the centre with the overall topic title, adding branches and subbranches for themes and subsidiary topics. 'Basic' maps consist of a branched hierarchy overwritten with key words (e.g. shaded portion above). Connections should be indicated with arrows: numbering and abbreviations are encouraged. To aid recall and creativity, Buzan and Buzan (2009) recommend use of colour, different fonts, three-dimensional doodles and other forms of emphasis (e.g. non-shaded portion above).

Printing presentation slides – use, for example, the 'Handouts' format on PowerPoint with several slides per page (e.g. Fig. 4.1) to help your note-making. Use the 'Black and White' option on the Print menu to avoid wasting ink on printing coloured backgrounds. If you wish to use colour, remember that slides can be difficult to read if printed in small format. Always print a sample page before printing the whole lecture.

lecture. On the other hand, typing notes tends to be a linear process – it can be more difficult to move backwards and forwards through your notes to make additional comments and amendments. Also, you need to avoid distractions (switch off the WiFi on your device so that you don't receive alerts for emails, social media, etc.).

Whatever technique you use, do not try to transcribe the lecturer's words, except when an important definition or example is being given, or when the lecturer has made it clear that he/she is dictating. Listen first, then summarise in your own words – you need to *make* your own notes, not just take down the lecturer's words. This is especially true when making notes using a laptop or tablet. Your goal should be to capture the structure and reasoning behind the lecturer's approach in as few words and phrases as possible.

At this stage, follow the lecturer's sequence of delivery. Use headings and leave plenty of space for later annotation, but do not worry too much about being too tidy – it is most important that you get down the appropriate information in a readable form.

Abbreviations can be useful to help you to save time when notetaking. As well as standard and well-known symbols (Box 4.1), you can also make up your own contractions relevant to the context. For example, if a lecturer is repeatedly mentioning photosynthesis, you might write 'PS' instead of the full term.

Make sure you note down any references to specific texts and take special care to ensure the accuracy of definitions and numerical examples. If the lecturer repeats or otherwise emphasises a point, highlight (for example, by underlining) or make a margin note of this – it could come in useful when revising. If there is something you do not understand, ask at the end of the lecture, or arrange to discuss the matter later if there is no time to deal with it then. Tutorials may provide an additional forum for discussing course topics.

As soon as possible after each lecture, work through your notes, tidying them up and adding detail where necessary. Add emphasis to any headings you have made, so that the structure is clearer. Use the left-hand margin to summarise, or to add key points. If you feel it would be more logical for your purposes, change the order. Compare your notes with material in a textbook and correct any inconsistencies. Make notes from, or photocopy, any useful material you see in textbooks, ready for revision.

Obtaining information from books and journal papers

As with lectures, you can optimise your learning from written resources through active note-making as you read, and many of the general points noted above apply equally to text-based material. There are a couple of supplementary approaches, depending on the purpose of your reading, as described below.

Adjusting to the different styles of your lecturers

– recognise that different approaches to lecture delivery demand different approaches to notetaking. For example, if a lecturer seems to tell lots of anecdotes or spends much of the time on examples during a lecture, do not switch off – you still need to be listening carefully to recognise the important take-home messages. Similarly, if a lecture includes a section consisting mainly of images, you should still try to take notes – names of organisms, locations, key features, even quick sketches. These will help prompt your memory when revising. Do not be deterred by lecturers' idiosyncrasies; in every case you still need to focus and take useful notes (see also Box 3.2).

Scanning

Good reading skills are vital when making notes from written sources. When consulting a new source for specific information, the first thing you need to do is to orientate yourself by understanding the text's scope and structure. This is often called 'scanning'.

- **For books**, this might involve a quick inspection of the contents section, a check on how each chapter is constructed, and noting, for example, whether the book has a glossary that might be useful. Once you are familiar with the structure and layout, then you might either go directly to the appropriate chapter or section, or consult the index for specific aspects.
- **For journal publications** (scientific papers, etc.), the best idea is to read the Abstract before you consult specific text, figures or tables, as this should summarise the essential methods and findings. The paper's layout thereafter will probably involve the same component sections ('IMRaD', p. 43), but occasionally in a journal-specific order. Referencing systems may also differ, depending on the journal's style (see p. 37).

Box 4.1 How to use abbreviations in notetaking

The following symbols are commonly used in the sciences.

\because	because	\equiv, \neq	equivalent, not equivalent to
\propto	is proportional to	$<, >$	smaller than, bigger than
\rightarrow	leads to, into	\gg	much bigger than
\leftarrow	comes from, from	[X]	concentration of X
$\rightarrow \rightarrow$	involves several processes in a sequence	Σ	sum
$1^\circ, 2^\circ$	primary, secondary (etc.)	Δ	change
\approx, \cong	approximately, roughly equal to	f	function
$=, \neq$	equals, not equal to	$\#$	number
		∞	infinity, infinite

Further general abbreviations can be found at <https://public.oed.com/how-to-use-the-oed/abbreviations/> and some relevant to biology at <https://www.allacronyms.com/biology/abbreviations>.

Focussing your study on your objectives

– reading around your subject is worthwhile and rewarding, but if your time is limited, it is important to identify a purpose for your reading and note-making. Ideally, this should relate to specific learning outcomes or assessments.

Scanning effectively – you need to stay focused on your key words; otherwise you may be distracted by apparently interesting but less relevant material.

Spotting sequences – writers often number their points (firstly, secondly, thirdly, etc.) and looking for these words in the text can help you skim it quickly.

Making sure you have all the details – when taking notes from a text or journal paper: (a) always take full details of the source, as described in Chapter 5; (b) if copying word-for-word make sure you indicate this using ‘speech marks’ – take special care to ensure you (i) do not alter the original wording and also (ii) quote the source in full, if used in an essay or other assignment.

At the next stage, when you are scanning through the text to find material relevant to your purpose, it is useful to have a good understanding of how most writing is structured. Both paragraphs and larger pieces of writing generally include an introduction, a main body and a conclusion. The introductory or ‘topic’ sentence/paragraph should orientate you to the material that will follow; the main body expands on the topic sentence, with explanation and examples; and the final or ‘terminator’ sentence/paragraph provides a conclusion and/or a link to the next part.

KEY POINT Working out how a piece of writing is structured will help you to scan quickly for relevance, understand the content faster and get to the material of interest as soon as possible.

Depending on your objective, you may have a specific key word or phrase you are searching for, or you may wish to read paragraphs on a specific topic. As well as an index, most biosciences textbooks use systems of headings and subheadings which facilitate such searches. With digital and online resources, you can press the **Control** and **F** keys together to access a ‘find’ dialog box that will speed the process of locating a key word – however, take care to enter correctly spelled and relevant words, using alternatives if an initial search draws a blank.

Finally, you need to read through the content and decide on the salient points. These will obviously depend on the task you are carrying out. When making notes, it is vital that you distinguish between your own paraphrasing or summarising of the text and any situations where your notes are direct quotes, or you may be guilty of plagiarism (see p. 42). If transcribing text word-for-word, always show that the text is taken directly from the original source by using quotation marks to highlight the transcribed words and, in addition, always give the full citation details of the source (pp. 38–40) at the same time.

Definitions

Quoting – this involves using text directly from your source and demonstrating this clearly by using quotation marks (and sometimes italics). If you wish to miss words out, use ellipsis punctuation marks (...), as shown in this example: *Wilson and Brand (2020) concluded: 'this indicates that sample size was small in this study ... but that this was acceptable in view of the low variability encountered.'* Here, the words 'because of financial constraints' have been omitted as irrelevant to the author's main point. For longer quotes (say 40 words or more), create a separate paragraph of text with a citation, usually given at the end of the quotation.

Summarising – this is taking the essence from a text and expressing it briefly in your own words. For example: *Other researchers defend the use of small sample sizes (e.g. Wilson and Brand, 2020).*

Paraphrasing – this is to quote others' ideas or points by restating them in different words. It is different from summary in that you may include more detail. For example: *Wilson and Brand (2020), based on their studies of the snail population in Arran, take the view that small sample size may be irrelevant when variability is low.*

In all cases above, note the citation of a relevant reference, in this case using Harvard style (author names and date, p. 38). Full details of the source would be provided in a reference or bibliography section (see p. 38). See also Chapter 5 (especially Box 5.1) in relation to plagiarism.

Learning online – use of a virtual learning environment (VLE) as a platform to provide learning, teaching and assessment resources is detailed in Chapter 3.

Skimming

This is a valuable way to gain the maximum amount of information in the minimum amount of time, by reading as little of a text as is required. Essentially, the technique (also termed 'surveying') requires you to look at the *structure* of the text, rather than the *detail*. In essence, you are trying to see the writer's original plan and the purpose behind each part of the text. Look over the whole of the piece first, to gain an overview of its scope and organisation. Where present, headings provide an obvious clue to structure. Next, look for the 'topic sentence' in each paragraph (Box 10.1) – typically the first sentence. You might then decide that the paragraph contains a definition that is important to note, or it may contain examples, so may not be worth reading in detail for your purpose.

When you have found relevant material, note-making fulfils the vital purpose of helping you understand and remember the information. If you simply read it passively, either directly or from a photocopy or print-out, you are at risk of accomplishing neither. The act of paraphrasing (using different words to give the same meaning) during note-making makes you think about the meaning and express this for yourself. It is an important active learning technique. A popular method of describing skimming and note-making is called the SQ3R technique (Box 4.2).

KEY POINT Obtaining information and understanding it are distinct parts of the process of learning. As discussed in Chapter 3 (Table 3.1), you must be able to do more than recall facts to succeed.

Methods for finding and evaluating texts and articles are discussed further in Chapters 5 and 6.

Using Internet resources

Most of the general points about learning from books and research papers also apply to material published on the Internet. Perhaps the most significant additional aspect of using the Internet as a learning resource is in relation to searching for appropriate material. The Internet enables you to access millions of sites containing text, images and links to other sites, and you will need to carefully consider your strategies for locating and then evaluating what you find.

KEY POINT In contrast to scientific publications (Box 12.2) most of the material published on the Internet has not been subject to peer review. It is essential that you consider the authority and veracity of anything that you find (p. 43 provides practical advice on how to evaluate Internet resources).

Some useful approaches to optimise your searching for Internet resources include:

- **For a comprehensive search**, use a variety of tools including search engines – both general (for example, Google) and academic-related (for example, e.g. Google Scholar) – and subject portals or directories. Box 4.3 gives advice on searches using Google.

Box 4.2 How to use the SQ3R technique for skimming texts

1. **Survey.** Gain a quick overview of the contents of the book or chapter, perhaps by rapidly reading the contents page or headings.
2. **Question.** Ask yourself what the material covers and how precisely it relates to your study objectives. Write down your answers.
3. **Read.** Now read the text, paying attention to the ways it addresses your key questions.
4. **Recall.** Recite to yourself what has been stated every few paragraphs. Write summary notes to further strengthen your learning, paraphrasing the text rather than copying it. This form of self-assessment can enhance learning (Chapter 3).
5. **Review.** Think about what you have read and/or review your notes as a whole. Consider where it fits into the 'bigger picture'.

- **For a complex, finely specified search,** employ Boolean operators and other tools to refine your keywords as fully as possible (Box 4.3). Some search engines allow you to include and exclude terms or restrict by date.
- **Use 'cascading' searching** when available – this is searching within the results of a previous search.
- **Use advanced search facilities** to limit your search, where possible, to the type of medium you are looking for (for example, graphics, video), language, sites in a specific country (for example, UK) or to a subject area (for example, news only).

However well defined your search is, you will still need to evaluate the information obtained (see Chapter 5).

In addition to using search engines, you may find it useful to consult the websites of scholarly organisations, including:

- **Libraries, publishers and commercial organisations.** Your university library is likely to subscribe to one or more academic databases providing access to journal articles; these include IngentaConnect (<http://www.ingentaconnect.com/>) Web of Science (<http://webofknowledge.com/>) and Science Direct (<http://www.sciencedirect.com/>). A password is usually required; consult your library staff for further details. Some scientific database sites give free access, without subscription or password; for example, the National Center for Biotechnology Information (USA) (<http://www.ncbi.nlm.nih.gov/>). Publishers such as Pearson (<http://www.pearsoned.com/>) and booksellers such as Amazon (<http://www.amazon.com/>) provide online catalogues and e-commerce sites that can be useful sources of information.
- **Online journals and e-books.** A number of traditional journals have websites. You can keep up-to-date by visiting the websites of *Nature* (<http://www.nature.com/>), *New Scientist* (<http://www.newscientist.com/>) and *Scientific American* (<http://www.scientificamerican.com/>). Some scientific societies make their journals and other publications available via their websites, for example, the American Society for Microbiology, at: <http://www.asm.org/>. Journals solely published in electronic format are also available, often requiring a password for access; check whether your institute is a subscriber. The directory of open access journals (at: <http://www.doaj.org/>) provides a comprehensive list of all open access (free, without subscription) journals.

Box 4.3 How to get the most from Google searches

Google (<http://www.google.com>) has become the search engine of choice for millions of people, due to its simplicity and effectiveness. However, you may be able to improve your searches by understanding its default settings and how they can be changed.

- **Download the Google toolbar to your browser.** This is available from the Google homepage and will give you quick access to the Google search facility.
- **Understand how standard operators are used.** For combinations of keywords, Google uses the minus operator ‘-’ instead of NOT (exclude) and ‘+’ instead of AND (include). Since Google usually ignores small words (‘stop words’ such as *in* or *the*), use ‘+’ to include them in a search. Where no operator is specified, Google assumes that you are looking for both terms (i.e. ‘+’ is default). If you want to search for alternative words, you can use ‘OR’ (e.g. *sulphur OR sulfur*). Google does not allow brackets and also ignores most punctuation marks.
- **While wildcard truncation of words using ‘*’ is not allowed, you can use ‘*’ to replace a whole word (or number).** For example, if you type the phrase “a virus is approximately * nanometres” (UK spelling) your results will give you results for Web pages where the wildcard is replaced by a number.
- **Search for exact wording.** By placing text in double inverted commas (“”), you can ensure that only websites with this exact phrasing will appear at the head of your search results.
- **Search within your results to improve the outcome.** If your first search has produced a large number of results, use the *Search within results* option near the bottom of each page to type in a further word or phrase.
- **Search for words within the title of a Web page.** Use the command *intitle:* to find a Web page, for example *intitle: “tissue culture”* returns Web pages with this phrase in the title (note that phrases must always be in double speech marks, not single quotes).
- **Search within a website.** Use the *site:* command to locate words/phrases on a specific website, for example *site:unicef.org dysentery* returns only those results for this disease on the UNICEF Website (*unicef.org*). Pressing *Control+F* when visiting a Web page will give you a pop-up search window.
- **Locate definitions, synonyms and spellings.** The operator *define:* enables you to find the meaning of a word. If you are unsure as to the spelling of a word, try each possibility: Google will usually return more results for the correct spelling and will often also prompt you with the correct spelling (*Did you mean. . . ?*).
- **Find similar Web pages.** Simply click the *Similar pages* option at the end of a Google search result to list other sites (note that these sites will not necessarily include the term(s) searched for).
- **If a hyperlink is unavailable, try the cached (stored) page.** Clicking on *Cached* at the end of a particular result should take you to the stored page, with the additional useful feature that the search term(s) will be highlighted.
- **Use the calculator functions.** Simply enter a calculation and press *Enter* to display the result, for example ‘10 + (2*4)’ returns 18. The calculator function can also carry out simple interconversion of units, e.g. ‘2 feet 6 inches in metres’ returns 0.762 (see Box 28.1 for interconversion factors between SI and non-SI units).
- **Try out the advanced search features.** In addition to the standard operators, these include the ability to specify the number of results per page (e.g. 50, to reduce the use of the *next* button), language (e.g. English), file format (e.g. for PDF files), recently updated Web pages (e.g. past three months) and usage (e.g. free to use/share).
- **Find non-text material.** These include images, video and maps – always check that any material you use is not subject to copyright limitations (p. 42).
- **Use Google alerts to keep up to date.** This function enables you to receive regular updated searches by email. Look for the ‘alerts’ feature available within the Google apps icon at <http://www.google.com/>.
- **Use Google Scholar to find articles and papers.** Go to <http://scholar.google.com/> and type in either the general topic or specific details for a particular article, e.g. author names or words from the title. Results show titles/authors of articles, with links to either the full article, abstract or citation. A useful feature is the *Cited by . . .* link, taking you to those papers that have cited the article in their bibliography and enabling you to carry out *forward citation searching* to locate more recent papers. Also try out the advanced scholar search features to limit your search to a particular author, journal, date or subject area. However, you should note that Google Scholar provides only a basic search facility to easily accessible articles and should not be viewed as a replacement for your library’s electronic journal holdings and searching software. For example, if you find the title of a paper via Google Scholar, you may be able to locate the electronic version through your own library’s databases, or request it via interlibrary loan (p. 37). Another significant limitation is that older (more cited) sources are typically listed first. ‘Use Advanced’ Search to find sources between years X and Y.
- **Use Google Earth to explore locations.** This allows you to zoom in on satellite images to find locations.

Understanding open access

publication – this is a growing movement, pioneered by the Public Library of Science (PLOS). Typically authors of scholarly articles, or their institutions, pay for all costs required for online publishing, thereby providing free and unrestricted online access to all potential readers. For an example of an open access journal, see: <http://www.plosbiology.org>.

Finding a website if a URL has changed

– make a keyword search using a search engine to find a particular site if the URL information you have does not lead you to an active page. Alternatively, use the Wayback Machine at: <http://www.archive.org> to locate an earlier version of a URL within the Internet Archive.

- **Data and images.** Archives of text material, video clips and photographs can be accessed, and much of the material is readily available. The CSIRO Science Image gallery (<https://www.scienceimage.csiro.au/>) is a good example. When downloading such material, you should always (i) check that you are not breaching copyright and (ii) avoid potential plagiarism by giving the full citation of the source (pp. 38–40), if you use such images in an assignment.
- **Biological institutions.** Many museums, botanic gardens, zoos, culture collections, scientific societies and other biological institutions around the world are now online. Use their sites to obtain specific information about collections, resources, etc. They frequently provide links to other relevant sites or topics, for example, the Biochemical Society (<https://www.biochemistry.org>) or the Microbiology Society (<http://microbiologysociety.org/>).
- **Databases.** In addition to those covering the scientific literature, others focus on specific topics (see, for example, https://en.wikipedia.org/wiki/List_of_biological_databases). Box 64.2 provides further guidance on using databases in the important field of bioinformatics. Examples of nucleic acid databases include the Rutgers University database (<http://ndbserver.rutgers.edu>) and the FBI CODIS database, used in forensic DNA analysis (search for ‘biometric analysis’ at <http://www.fbi.gov/>).

Focussing on relevant material

One of the benefits of exploring books, academic journals and websites is finding interesting material that fires your enthusiasm for the subject. Some of this information might be used, for example, to add examples or ‘colour’ to an essay (which might gain you credit). However, it is important to make your studying effective by ensuring that it fits with the expectations of your lecturers. These will be explained in outline terms in the learning objectives/outcomes for the module and course. Also relevant is the assessment profile, which will indicate how understanding you gain might be tested. Both these aspects of study are discussed in detail in Chapter 9.

KEY POINT When consulting learning resources, always keep your aims in mind. Try not to get too distracted when browsing bookshelves or Web articles – but if you do find information that could be relevant elsewhere, make sure you take a note of what it was and where you found it.

Text reference

Buzan, T. and Buzan, B. (2009) *The Mind Map Book: Unlock Your Creativity, Boost Your Memory, Change Your Life*. Pearson, Harlow.

Sources for further study

Anon. Evaluating Web Resources. Available <https://www.library.kent.edu/criteria-evaluating-web-resources>
Last accessed 26/04/2021.

Anon. Mind Map. Available http://en.wikipedia.org/wiki/Mind_map
Last accessed 26/04/2021.

STUDY EXERCISES

4.1 Experiment with a new note-making technique.

If you have not tried the pattern or mind-mapping methods (Figs 4.2 and 4.3), try them both out to see how they might work for you. Research the methods first by consulting appropriate books or websites.

4.2 Try out the SQ3R technique.

The next time you need to obtain information from a text, compare this method (Box 4.2) with others you may have adopted in the past. Is it faster, and does it aid your ability to recall the information, based on self-evaluation?

4.3 Compare results from a variety of search engines and databases.

First, think of an appropriate biological keyword or phrase (e.g. a species name) and enter this into several search engines (e.g. Google, DuckDuckGo), academic search engines (e.g. Google Scholar) and searchable databases (e.g. Core). Compare the outcomes to reveal the strengths and weaknesses of each. Work with a 'study buddy' to compare different searches on a quantitative (i.e. number of hits) and qualitative (quality of hits) basis.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

5 Locating and citing published information

Browsing in a library – this may turn up interesting material, but remember that the books on the shelves are those *not* currently out on loan. Almost by definition, the latter may be more up to date and useful. To find out a library's full holding of books in any subject area, search the online catalogue. An online search will also show any e-books in the library collection – many books will only be available in this form.

Example The book *The Selfish Gene* by Richard Dawkins (1976; Oxford University Press) is likely to be classified as follows:

Dewey Decimal system: 591.51

where 591	refers to zoology
591.5	refers to ecology of animals
591.51	refers to habits and behaviour patterns

Library of Congress system: QL751

where Q	refers to science
QL	refers to zoology
QL75	refers to animal behaviour
QL751	refers to general works and treatises

Using reliable websites – there are several high quality scientific websites that are free to use ('open access'), including:

Biomedcentral (www.biomedcentral.com) – BMC journals.

Core (<https://core.ac.uk>) – database of open access papers.

Plos (<https://plos.com>) – Plos biology journal.

Pubmedcentral (<https://www.ncbi.nlm.nih.gov/pmc>) – free archive of over 6 million biomedical and life science papers.

The ability to find appropriate scientific information is a skill required for many exercises in your degree program. You will need to research facts and published findings as part of writing essays, literature reviews and project introductions, and when amplifying your lecture notes and revising for exams. You must also learn how to follow scientific convention in referring to (citing) source material as the authority for the statements you have made.

Finding sources of information

Background material for essays and revision

You are more likely to make use of books and reviews for introductory modules, while scientific papers will be used in advanced modules, project work and dissertations. If a lecturer or tutor recommends a particular book, check if it is available in the library, either online or in print. Library staff will generally be happy to assist with any queries. If you want to find out what information your library has on a specified topic, check the online catalogue, which usually covers both books and journals (for reviews). You will also be able to search by author or by key words.

There are two main systems used by libraries to classify books: the Dewey Decimal system and the Library of Congress system. Libraries differ in the way they employ these systems, especially by adding further numbers and letters after the standard classification marks to signify, for example, shelving position or edition number. Consult the library website/staff to see if there is an online guide or booklet explaining how their system works.

The Internet is an ever-expanding resource for general and specific information (see Chapter 6). Websites fall into analogous categories to those in the printed literature: there are sites with original information (primary sources, p. 43), sites that review information (secondary sources, p. 43) and bibliographic sites – (tertiary sources p. 43). While there are some highly regarded science websites, you need to check to confirm that information you find online is accurate and scholarly (see p. 43 for how to evaluate information on the Internet). One problem is that websites may be frequently updated, so information present when you first look may be altered or even absent when the site is next consulted. Furthermore, little of the general information on the Internet has been monitored or refereed. Another disadvantage is that the website may not state the origin of the material, who wrote it or when it was written (p. 43).

Material for literature surveys and project work

You will probably need to consult primary sources ('papers'). If you are starting a new research project or writing a report from scratch, you can build up a core of relevant papers by using the following methods:

- **Asking around:** supervisors or their postgraduate students will almost certainly be able to supply you with a reference or two that will start you off.
- **Searching online science databases:** these enable you to undertake comprehensive searches of the scientific literature, and are essential for identifying relevant articles. Most databases enable you to save a particular search, and many have hyperlinks taking you to individual sources, which can then be saved.

Researching a new topic – reading reviews (secondary sources, p. 43) can provide you with a useful overview at the start of a new project. Some databases will have an option that allows you to search just for review articles.

Using science databases – your library's website will provide you with access to a range of links to online databases, which may be worth bookmarking on your Web browser. Examples of resources especially useful to bioscientists include:

- *ISI Web of Science*, including the *Science Citation Index*
- *Ingenta Connect*
- *Medline/PubMed*
- *ScienceDirect*
- *Scopus*

Most of these electronic databases operate on a subscription basis and may require a username and password (often the same as your student login). Consult library staff or your library's website if you have difficulty accessing or using databases.

- **Consulting the bibliography/references of other papers in your collection:** this is an important way of finding the key papers in your field. In effect, you are taking advantage of the fact that another researcher has already done all the hard work.
- **Referring to 'current awareness' online databases:** these provide regular listings of article details (title, authors, source, author address) arranged by subject and cross-referenced by subject and author. Current awareness databases cover a wider range of primary literature than could ever be available in any one library. Examples relevant to the biosciences include: *Current Contents Connect* and *Current Advances* (Elsevier). Most online databases and scientific journals offer an 'alert' service and will email registered users with updates based on saved search criteria. Check your library's website to see which of these databases and services are available to you.
- **Using the *Science Citation Index (SCI)*:** this is a valuable source for exploring the published literature in a given field, because it lets you see who has cited a given paper; in effect, SCI allows you to move forward through the literature from an existing reference. The Index is available online via the Web of Science.
- **Using *Google Scholar*:** this enables you to find highly cited publications on particular topics (keywords used in search) – such publications are likely to have had more impact on a particular research field than those that are rarely cited. As with the SCI, you can work forwards, identifying recent publications that cite a particular key paper from earlier years.

Specialised information

You may need to consult reference works, such as encyclopaedias, maps and books providing specialised information, such as the properties of a chemical, the location of a research site or the biography of a particular scientist. Much of this is now available online (consult your library's Web pages or information service).

Two general sources worth noting are:

- **The *CRC Handbook of Chemistry and Physics*** (Rumble, 2020) – also available online at: www.hbcponline.com: the Chemical Rubber Company's publication (affectionately known as the 'Rubber Bible') giving all manner of physical constants, radioisotope half-lives, etc.
- **The *Merck Index Online*** (Royal Society of Chemistry, 2020), which gives useful information about organic chemicals, for example, solubility, whether poisonous, etc.

Obtaining and organising your research papers

Obtaining a copy

It is usually more convenient to have personal copies of key research articles in electronic or print format for direct consultation when working in a laboratory or writing. The simplest approach is to download an electronic copy (for example, a PDF file). Alternatively you can photocopy a printed copy of the article. For academic purposes, this is normally acceptable within copyright law. If your library does not subscribe to the journal, it may be possible for them to obtain a copy from another library (an 'inter-library loan'). If the latter, you will have to provide full bibliographic details of

Definitions

e-book – a book published online.

e-journal – a journal published online, consisting of articles structured in the same way as a paper-based journal. A valid username and password may be required for access (arranged via your library, if it subscribes to the e-journal).

Journal/periodical/serial – any publication issued at regular intervals. In biosciences, journals usually contain papers (articles) describing original research findings and reviews of literature.

Monograph – a specialised book focussed on a single topic.

The primary literature – this comprises original research papers, published in specialist scientific periodicals. Certain prestigious general journals (e.g. Nature) contain important new advances from a wide subject area.

Definitions

Abstracts – shortened versions of papers, often those read at scientific meetings. These may later appear in the literature as full papers.

Bibliography – a summary of the published work in a defined subject area.

Proceedings – a compilation of written versions of papers read at a scientific meeting on a specific topic.

Review – an article in which recent advances in a specific area are outlined and discussed.

Trying alternative methods of receiving information

– RSS (really simple syndication) feeds and email updates from publishers are increasingly used to provide automated information services to academic clients, for example, by supplying links to relevant contents of new editions of online journals or to updates from scientific organisations.

the paper and where it was cited, as well as signing a copyright clearance statement concerning your use of the copy.

If you are unable to obtain a copy from your library, or via inter-library loan, then you might consider emailing or writing to the author to request a copy (use a Google search to find an author's address and email details). In some cases, authors may also have permission from the publisher to place a copy of an article on their institutional Web page or in their institution's research repository (these are usually publicly accessible).

Organising papers

Although the number of papers you accumulate in electronic and/or hard copy formats may be small to start with, it is worth putting some thought into their storage and indexing before your collection becomes disorganised and unmanageable. Few things are more frustrating than not being able to lay your hands on a vital piece of information when required, and this can seriously disrupt your flow when writing or revising.

Indexing your research papers

Whether you have stored downloaded files electronically, printed a hard copy or have simply noted the bibliographic details of a reference, you will need to 'index' each resource. This is valuable for the following reasons:

- you will need the bibliographic information for creating a 'reference list', citing sources for an assignment or report
- if your index also has database features, this can enable you to search for key words or authors
- depending on the indexing system used, you can add comments about the reference that may be useful at a later time, for example, when writing an introduction or conclusion for an assignment.

The simplest way to create an index system is to put the details on reference cards. However, Bibliographic Management Software (BMS) can be more convenient and faster to sort, once the bibliographic information has been entered. BMS enables you to create your own 'library' of references to which you can attach a wide range of files; commonly used BMS tools include Zotero (free), EndNote, RefWorks and Mendeley – check with your library to see if your university has an institutional subscription. If you feel that you do not need commercial software, a spreadsheet with basic sorting functions (Chapter 72) may meet your needs.

If you are likely to store lots of references and other electronic resources digitally, then you should consider carefully how this information is kept, for example, by choosing file names that indicate what the file contains and that will facilitate searching/sorting.

Making citations in text

It is particularly important to cite (refer to) all sources of information ('references') used in your work – this will demonstrate to assessors that you have used appropriate source material and it will also avoid any possible accusations of plagiarism (using another person's work without appropriate acknowledgement, p. 41).

There are two main ways of citing articles and creating a list of sources (also referred to as 'references', 'literature cited', or 'bibliography' – the latter term is often used to include sources consulted but not cited).

Understanding copyright law – in Europe, copyright regulations were harmonised in 1993 (Directive 93/98/EEC) to allow literary copyright for 70 years after the death of an author and typographical copyright for 25 years after publication. This was implemented in the UK in 1996, where, in addition, the Copyright, Designs and Patents Act (1988) allows the Copyright Licensing Agency to license institutions so that lecturers, students and researchers may take copies for teaching and personal research purposes – no more than a single article per journal issue, one chapter of a book, or extracts to a total of 5% of a book. The substance of this act is likely to be maintained after 2021.

Storing printed research papers – these can easily be kept in alphabetical order within filing boxes or drawers, but if your collection is likely to grow large, it will need to be refiled as it outgrows the storage space. You may therefore wish to add an ‘accession number’ to the record you keep in your database, and then file the papers in sequence according to this as they accumulate.

Using a bibliographic management system (BMS) to organise your papers and present your references – for those with large numbers of sources in their collection, and who may wish to produce lists of selected references in particular formats, e.g. for inclusion in a project report or journal paper, a BMS may be worth the investment of time required to create a personal reference database. Appropriate bibliographic data must first be entered into fields within the system (most online databases enable the exporting of files directly into your BMS; content and files can also be added manually). The database can then be searched and used to create customised lists of selected references in appropriate citation styles.

The Harvard system

For each citation, the author name(s) and the date of publication are given at the relevant point in the text. The References section is then organised alphabetically and by date of publication for papers with the same authors. Formats normally adopted are, for example, ‘Smith and Jones (2013) stated that . . .’ or ‘it has been shown that . . . (Smith and Jones, 2013)’. Lists of references within parentheses are separated by semicolons, for example ‘(Smith and Jones, 2013; Jones and Smith, 2018)’, normally in order of date of publication. To avoid repetition within the same paragraph, an approach such as ‘the investigations of Smith and Jones indicated that’ could be used following an initial citation of the paper. Where there are more than two authors it is usual to write ‘*et al.*’; this stands for the Latin *et alia* meaning ‘and others’. If citing more than one paper with the same authors, put, for example, ‘Smith and Jones (2013, 2020)’ and if papers by a given set of authors appeared in the same year, letter them (for example, Smith and Jones, 2020a; 2020b).

The numerical or Vancouver system

Papers are cited via a superscript or bracketed reference number inserted at the appropriate point. Normal format would be, for example: ‘DNA sequences^{4,5} have shown that . . .’ or ‘Jones [55,82] has claimed that . . .’. Repeated citations use the number from the first citation. In the true numerical method (for example, as in *Nature*), numbers are allocated by order of citation in the text, but in the alpha-numerical method (for example, the *Annual Review* series), the references are first ordered alphabetically in the Literature Cited section, then numbered, and it is this number that is used in the text. Note that with this method, adding or removing references is tedious, so the numbering should be done when the text has been finalised.

KEY POINT The main advantages of the Harvard system are that the reader might recognise the paper being referred to and that it is easily expanded if extra references are added. The main advantages of the Vancouver system are that it aids text flow and reduces length.

Listing your sources

Whichever citation method is used to refer to sources within your text, comprehensive details are required for the References section, so that the reader has enough information to find the reference easily. Sources cited should be listed in alphabetical order with the priority: first author, subsequent author(s), date. Unfortunately, in terms of punctuation and layout, there are almost as many ways of citing papers as there are journals! Your department may specify an exact format for project work; if not, then you should decide on a style and be consistent – if you do not pay attention to the details of citation you may lose marks. Take special care with the following aspects:

- **Authors and editors:** give details of *all* authors and editors in your list of references, even if given as ‘*et al.*’ in the text.
- **Abbreviations for journals:** while standard abbreviations for the titles of journals are often used in publications (to save space), it is a good idea to give the whole title, if possible.

Example Incorporating references in text – this sample shows how you might embed citations in text using the Harvard approach:

‘... Brookes *et al.* (2020) proposed that protein A216 was involved in the degradation process. However, others have disputed this notion (Scott and Davis, 2007; Harley, 2008, 2010). Patel (2020a 2020b) found that A216 is inactivated at pH values less than 5; while several authors (e.g. Hamilton, 2018; Drummond and Stewart, 2019) also report that its activity is strongly dependent on Ca^{2+} concentration . . . ’

Example Incorporating references in text – this sample shows how you might embed citations in text using the Vancouver approach:

‘... Brookes *et al.* proposed that protein A216 was involved in the degradation process¹. However, others have disputed this notion^{2–4}. Patel^{5,6} found that A216 is inactivated at pH values less than 5; while several authors^{7,8} also report that its activity is strongly dependent on Ca^{2+} concentration . . . ’

Examples

Paper in journal:

Smith, A. B., Jones, C.D. and Professor, A. (2018). Innovative results concerning our research interest. *Journal of New Results*, **11**, 234–5.

Book:

Smith, A. B. (2019). *Summary of My Life's Work*. Megadosh Publishing Corp., Bigcity. ISBN 123-4-56-789123-4.

Chapter in edited book:

Jones, C. D. and Smith, A. B. (2020). Earth-shattering research from our laboratory. In: *Research Compendium 1998* (ed. A. Professor), pp. 123–456. Bigbucks Press, Booktown.

Thesis:

Smith, A. B. (2012). *Investigations on my Favourite Topic*. PhD thesis, University of Life, Fulchester.

- **Books:** the edition should always be specified, as contents may change between editions. Add, for example, ‘(5th edition)’ after the title of the book. You may be asked to give the International Standard Book Number (ISBN), a unique reference number for each book published.
- **Unsigned articles,** for example, unattributed newspaper articles and instruction manuals: refer to the author(s) in text and in the References section as ‘Anon.’.
- **Websites:** as with journal articles, there is no absolute format. You should follow departmental guidelines if these are provided, but if these are not available, we suggest providing author name(s) and date in the text when using the Harvard system (for example, Hacker, 2019), while in the References section giving the URL details in the following format: Hacker, A. (2019) *University of Anytown Homepage on Aardvarks*. Available: <http://www.myserver.ac.uk/homepage>. Last accessed 27/04/2021. In this example, the Web page was constructed in 2019, but accessed in April, 2020. If no author is identifiable, cite the sponsoring body (for example, University of Anytown, 2010), and if there is no author or sponsoring body, write ‘Anon.’ for ‘anonymous’, for example, Anon. (2010), and use Anon. as the ‘author’ in the References section. If the Web pages are undated, *either* use the ‘Last accessed’ date for citation and put no date after the author name(s) in the reference list, *or* cite as ‘no date’ (for example, Hacker, no date) and leave out a date after the author name(s) in the reference list – you should be consistent whichever option you choose.
- **Unread articles:** you may be forced to refer to a paper via another without having seen it. If possible, refer to another authority who has cited the paper, for example, ‘... Jones (1990), cited in Smith (2019), claimed that ...’. Alternatively, you could denote such references in the References section by an asterisk and add a short note to explain at the start of the list.
- **Personal communications:** information received in a letter, seminar or conversation can be referred to in the text as, for example, ‘... (Smith, pers. comm.)’. These citations are not generally listed in the References section of papers, though in a thesis you could give a list of personal communicants and their addresses.
- **Online material:** some articles are published solely online and others online ahead of publication in printed form. The article may be given a DOI (digital object identifier), allowing it to be cited and potentially tracked before and after it is allocated to a printed issue (see <http://www.doi.org/>). DOIs allow for Web page redirection by a central agency, and CrossRef (see <http://www.crossref.org>) is the official DOI registration organisation for scholarly and professional publications. DOIs can be used as ‘live’ hyperlinks in online articles, or cited in place of (and, when they become available, following) the volume and page numbers for the article, with the remainder of the details cited in the usual fashion: for example,

‘Smith, A. and Jones, B. (2019) Our latest important research in the form of a web-published article. *Online Biosciences* 8/2019 (p. 781). Published Online: 26 March 2019. DOI: 10.1083/mabi.200680019’.

Website:

Jones, A. B. (2020) *My Webpage on my Work in 2020*. Available: <http://www.jonesinfo.co.uk/work2020>.
Last accessed 27/04/2021.

If you are using a different referencing system from Harvard, consult Pears and Shields (2019) for further advice.

- **Italicised text:** always follow the italicisation shown in the examples above (for example, in journal titles); in handwritten work, indicate italics by underlining relevant text or numerals.

Text references

Pears, R. and Shields, G. (2019) *Cite Them Right! The Essential Referencing Guide*, 11th edn. Palgrave Macmillan, Basingstoke. Also available as an online resource, see http://www.citethemright_online.com/, with institutional subscription.

Royal Society of Chemistry (2020) Merck Index Online.
Available: <https://www.rsc.org/merck-index>
Last accessed 27/04/2021.

Rumble, J.R. (ed.) (2020) *CRC Handbook of Chemistry and Physics*, 100th edn. CRC Press, Boca Raton.

[Also check to see if your library has an institutional guide (a 'libguide') to citation and referencing.]

STUDY EXERCISES

5.1 Test your library skills. This exercise relies on the fact that most university-level libraries serving biosciences departments will take the scientific journal *Nature*. To help you answer these questions, it may be beneficial to attend a library induction session, if you have not already. Alternatively, the library's help or enquiry desks may be able to assist you if you are having problems.

- First, find out and provide the name of the classification system that your university uses for cataloguing its books and periodicals.
- Using your library's online cataloguing system find out the appropriate local classification number for the journal *Nature*.
- Where is the printed copy of *Nature* shelved in your library? (Your answer need refer only to most recent issues if some have been archived.)
- What is the exact title of the landmark papers in the following two volumes? (i) *Nature* **171**, 737–738 (1953); (ii) *Nature* **408**, 796–815 (2000).

5.2 Explore different methods of citing references. Go to the websites of three different journals in your subject area and from either their 'instructions to authors' or a recent issue, write down how they would print a typical citation for a multi-author journal paper in the 'references' or 'literature cited' section. When handwriting, indicate

italicised text with normal underline and bold text with wavy underline. Pay attention to punctuation. Compare these methods with each other, with the methods recommended on pp. 38–39 of this book and with the recommendation your department or your course handbook makes. Are they all the same?

5.3 Make website citations. Use a search engine to find an informative website that covers each of the following:

- The use of SI units.
- Information about pollination of a specific flower by bats.
- The different types of blood cells.

Indicate how you would cite each website at the end of an essay (follow your department's guidelines or use those in this chapter).

5.4 Compare the Harvard and Vancouver methods of citation. Pair up with a classmate. Each person should then pick one of the two main methods of citation and consider its pros and cons independently. Meet together and compare your lists. Given the choice, which method would you choose for (a) a hand-written essay; (b) a word-processed review; (c) an article in an academic journal, and why?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

6 Evaluating information

Example A Web search for the letters 'DNA' (e.g. using the search engine Google, p. 32), will reveal that this acronym appears in several hundred million websites. Not all of these deal with deoxyribonucleic acid – the listed websites include: *Doctors' Net Access*, a Web resource for American physicians; the website of the *Dermatology Nurses Association*; and that of *Delta Nu Alpha*, an international organisation for those working in the professions. These examples are easy to identify as irrelevant to research on 'DNA, the molecule', but considering the many websites that cover a given topic, which might contain the exact information you seek? How valid is the information? Is it biased towards one viewpoint or hypothesis? Does it represent current mainstream thinking on the topic? These are some of the issues that an evaluation of the information sources might deal with.

Definition

Plagiarism – the unacknowledged use of another's work as if it were one's own. In this definition, the concept of 'work' includes ideas, writing, data or inventions and not simply words; and the notion of 'use' does not only mean copy 'word for word', but also 'in substance' (i.e. a copy of the ideas involved). Use of another's work is only acceptable if you acknowledge the source (see Box 6.1).

Checking the reliability of information, assessing the relative value of different ideas and thinking critically are skills essential to the scientific approach. You will need to develop your abilities to evaluate information in this way because:

- **you will be faced with many sources of information**, from which you will need to select the most appropriate material
- **you may come across two conflicting sources of evidence** and may have to decide which is the more reliable
- **the accuracy and validity of a specific fact may be vital** to your work
- **you may doubt the quality of information** from a particular source
- **you may wish to check the original source** because you are not sure whether someone else is quoting it correctly.

KEY POINT Evaluating information and thinking critically are regarded as higher-order academic skills (Chapter 3). The ability to think deeply in this way is greatly valued in the biosciences and will consequently be assessed in coursework and exam questions (see Chapter 9).

Processing information

The process of evaluating and using information can be broken down into four stages:

1. **Selecting and obtaining material.** How to find sources is covered in Chapter 5. The Internet is often a first port of call if you wish to find something out quickly. For many websites, however, it can be difficult to verify the authenticity of the information given. Printed books and journals are important, but if you identify a source of this kind there may be delays in borrowing it or obtaining a photocopy. If the book or journal is available online, then downloading or printing sections or papers will be more convenient and faster.
2. **Assessing the content.** You will need to understand fully what has been written, including any technical terms and jargon used. Establish the relevance of the information to your needs and assure yourself that the data or conclusions have been presented in an unbiased way.
3. **Modifying the information.** In order to use the information, you may need to alter it to suit your needs. This may require you to make comparisons, interpret or summarise. Some sources may require translation. Some data may require mathematical transformation before they are useful. There is a chance of error in any of these processes and also a risk of plagiarism.
4. **Analysing.** This may be your own interpretation of the information presented, or an examination of the way the original author has used the information.

KEY POINT Advances in communications and information technology mean that we can now access almost limitless knowledge. Consequently, the ability to *evaluate* information has become an extremely important skill.

Box 6.1 How to avoid plagiarism and copyright infringement

• **Plagiarism** is defined on p. 41. Examples of plagiarism include:

- copying the work of a fellow student (past or present) and passing it off as your own
- using 'essay-writing services', such as those on offer on certain websites
- copying text or images from a source (book, journal article or website, for instance) and using this within your own work without acknowledgement
- quoting others' words without indicating who wrote or said them
- copying ideas and concepts from a source without acknowledgement, even if you paraphrase them.

Most students would accept that some of the above can be described as cheating. However, many students, especially at the start of their studies, are unaware of the academic rule that they must *always* acknowledge the originators of information, ideas and concepts, and that not doing so is regarded as a form of academic dishonesty. If you adopt the appropriate conventions that avoid such accusations, you will avoid accusations of plagiarism and you will also achieve higher marks for your work as it will fulfil the markers' expectations for academic writing.

Universities have a range of mechanisms for identifying plagiarism, from employing experienced and vigilant coursework markers and external examiners to analysing students' work using sophisticated software programs, such as Turnitin. In the latter, submitted text is compared to an extremely large global database of articles, websites, books and student assignments, with text matches shown side-by-side and as an overall 'similarity score', expressed as a percentage. A tutor can then view the output from Turnitin to form a judgement as to whether plagiarism is present in the work. If your university allows students to submit drafts to Turnitin, you can use this feature to check your work before final submission. Plagiarism is always punished severely when detected. Where copying a fellow student's work is concerned, penalties may include awarding a mark of zero to all involved – both the copier(s) and the person whose work has been copied (who is regarded as complicit in the crime). Further disciplinary measures may be taken in some instances. In severe cases, such as copying substantive parts of another's work within a thesis, a student may be dismissed from the university.

To avoid being accused of plagiarism, the remedies are relatively simple, as listed below:

- Make sure the work you present is always your own. If you have been studying alongside a classmate, or discussing how to tackle a particular problem with your peers, make sure you write alone when working on your assignments.

- Never be tempted to 'cut and paste' from websites or online documents. Read these carefully, decide what the important points are, express these *in your own words* and *provide literature citations to the original sources* (see Chapter 5).
- Take care when note-making. If you decide to copy text word-for-word, make sure you show this clearly in your notes with quotation marks. If you decide to make your own notes based on a source, make sure these paraphrase, rather than copy, phrases from the text. In both cases, write down full details of the source at the appropriate point in your notes.
- Place appropriate citations throughout your text where required. If you are unsure about when to do this, study reviews and articles in your subject area.
- Understand the differences between quotation, summarising and paraphrasing (p. 30). Apply the relevant layout conventions: show clearly where you are quoting directly from a source by using quotation marks, and *always* cite your source(s). Try not to rely too much on quotation as this may be regarded as a lack of ability to synthesise your own ideas.

• **Copyright** issues are often associated with plagiarism, and refer to the right to publish (and hence copy) original material, such as text, images and music. Copyright material is indicated by a symbol © and a date (see, for example, page iv of this book). Literary copyright is the aspect most relevant to students in their academic studies. UK copyright law protects authors' rights for life and gives their estates rights for a further 70 years. Publishers have 'typographical copyright' that lasts for 25 years. This means that it is illegal to photocopy, scan or print out copyright material unless you have permission, or unless your copying is limited to an extent that could be considered 'fair dealing'. For educational purposes – private study or research – in a scientific context, this generally means:

- no more than 5% in total of a work
- one chapter of a book or 20% of a short book
- one article per volume of an academic journal
- one separate illustration or map.

You may only take one copy within the above limits, may not copy for others, and may not exceed these amounts *even if you own a copy of the original*. These rules also apply to Web-based materials, but sometimes you will find sites where the copyright is waived. Some copying may be licensed; you should consult your library's website or helpdesk to see whether it has access to licensed material. Up-to-date copyright information is generally provided on library websites/ VLE areas or posted close to library and departmental photocopiers.

Evaluating sources of information

One way of assessing the reliability of a piece of scientific information is to think about how it was obtained in the first place. Essentially, facts and ideas originate from someone's research or scholarship, whether they are numerical data, descriptions, concepts, or interpretations. Sources are divided into two main types:

Distinguishing between primary and secondary sources – try the 'IMRaD test'.

Many primary sources contain information in the order: Introduction, Materials and Methods, Results and Discussion. If you see this format, and particularly if *data* from an experiment, study or observation are presented without a citation to a different source, then you are probably reading a primary source.

Example If a journalist wrote an article about a new 'flesh-eating bug' for the *New York Times* that was based on an article in the *British Medical Journal*, the *New York Times* article would be the secondary source, while the *British Medical Journal* article would be the primary source.

Taking account of the changing nature of websites and wikis – by their very nature, these sources may change. This means that it is important to quote accurately from them and to give a 'Last accessed' date when citing (see p. 39).

Finding out about authors and provenance – these pieces of information are easy to find in most printed sources and may even be presented just below the title, for convenience. In the case of the Web, it may not be so easy to find what you want. Relevant clues can be obtained from 'home page' links and the header, body and footer information. For example, the domain (e.g. '.ac' for academic or '.com' for commercial) may be useful, while the use of the tilde symbol (~) in an address usually indicates a personal, rather than an institutional, website.

- 1. Primary sources** – those in which ideas and data are first communicated. The primary literature is generally published in the form of 'papers' (articles) in journals whether printed or online. These are usually refereed by experts in the academic peer group of the author, and they will check the accuracy and originality of the work and report their opinions back to the editors. This peer review system helps to maintain reliability, but it is not perfect. Books and, more rarely, websites and articles in magazines and newspapers, can also be primary sources but this depends on the nature of the information published rather than the medium. These sources are not formally refereed, although they may be read by editors and lawyers to check for errors and unsubstantiated or libellous allegations.
- 2. Secondary sources** – those which quote, adapt, interpret, translate, develop or otherwise use information drawn from primary sources. It is the act of quoting or paraphrasing that makes the source secondary, rather than the medium. Reviews are examples of secondary scientific sources, and books and magazine articles are often of this type.

When information is modified for use in a secondary source, alterations are likely to occur, whether intentional or unintentional. Most authors do not deliberately set out to change the meaning of the primary source, but they may unwittingly do so, for example, in changing text to avoid plagiarism or by oversimplification. Others may consciously or unconsciously exert bias in their reporting, for example, by quoting evidence that supports only one side of a debate. Therefore, the closer you can get to the primary source, the more reliable the information is likely to be. On the other hand, modification while creating a secondary source could involve correcting errors, or synthesising ideas and content from multiple sources.

Authorship and provenance

Clearly, much depends on who is writing the source and on what basis (i.e. who paid them?). Consequently, an important way of assessing sources is to investigate the ownership and provenance of the work (who and where it originated from, and why).

Can you identify who wrote the information? If it is signed or there is a 'by-line' showing who wrote it, you might be able to make a judgement on the quality of what you are reading. This may be a simple decision, if you know or can assume that the writer is an authority in the area; otherwise a little research might help (for example, by putting the name into a search engine). Of course, just because Professor X thinks something does not make it true. However, if you know that this opinion is backed up by years of research and experience, then you might take it a little more seriously than the thoughts of a school pupil. If an author is not cited, effectively nobody is taking responsibility for the content. Could there be a reason for this?

Assessing substance over presentation – just because the information is presented well (e.g. in a glossy magazine or particularly well-constructed website), this does not necessarily tell you much about its quality. Try to look below the surface, using the methods mentioned in this chapter.

Table 6.1 Checklist for assessing information in science. How reliable is the information you have been reading? The more ‘yes’ answers you can give below, the more trustworthy you can assume it to be

Assessing sources
<ul style="list-style-type: none"> • Can you identify the author’s name? • Can you determine what relevant qualifications he/she holds? • Can you say who employs the author? • Do you know who paid for the work to be done? • Is this a primary or secondary source? • Is the content original or derived from another source?
Evaluating information
<ul style="list-style-type: none"> • Have you checked a range of sources? • Is the information supported by relevant literature citation? • Is the age of the source likely to be important regarding the accuracy of the information? • Have you focused on the substance of the information presented rather than its packaging? • Is the information fact or opinion? • Have you checked for any logical fallacies in the arguments? • Does the language used indicate anything about the status of the information? • Have the errors associated with any numbers been taken into account? • Have the data been analysed using appropriate statistics? • Are any graphs constructed fairly?

Is the author’s place of work cited? This might tell you whether the facts or opinions given are based on an academic study. Is there a company with a vested interest behind the content? If the author works for a public body, there may be publication rules to follow and they may even have to submit their work to a publications committee before it is disseminated. They are certainly more likely to get into trouble if they include controversial material.

Evaluating facts and ideas

However reliable the source of a piece of information seems to be, it is probably a good idea to retain a slight degree of scepticism about the facts or ideas involved. Even information from impeccable primary sources may not be perfect – different approaches can give different outcomes, and interpretations can change with time and with further advances in knowledge. Table 6.1 provides a checklist to use when evaluating sources.

Critically examining facts and ideas is a complex task depending on the particular issues involved, and a number of different general approaches can be applied. You will need to decide which of the following general tips are useful in your specific case:

- **Make cross-referencing checks (triangulation)** – look at more than one source and compare what is said in each. The cross-referenced sources should be as independent as possible (for example, do not compare a primary source together with a secondary review based on it). If you find that all the sources give a similar picture, then you can be more confident about the reliability of the information.
- **Look at the extent and quality of citations** – if references are quoted, these indicate that a certain amount of research has been carried out beforehand, and that the ideas or results are based on genuine scholarship. If you are doubtful about the quality of the work, these references might be worth looking at. How up to date are they? Does their content really support what has been written, or have ideas and information been used selectively (‘cherry picking’)? Do they cite independent work, or is the author exclusively quoting their own work, or solely the work of one person?
- **Consider the age of the source** – the fact that a source is old is not necessarily a barrier to truth, but ideas and facts may have altered since the date of publication, and methods may have improved, especially in fast-moving fields such as molecular biology. Can you trace changes through time in the sources available to you? What key events or publications have forced any changes in the conclusions?
- **Try to distinguish fact from opinion** – to what extent has the author supported a given viewpoint? Have relevant facts been quoted, via literature citations or the author’s own researches? Are numerical data used to substantiate the points used? Are these reliable and can you verify the information, for example, by looking at the sources cited? Might the author have a reason for putting forward biased evidence to support a personal opinion?
- **Analyse the language used** – words and their use can be very revealing. Subjective wording might indicate a personal opinion rather than an objective conclusion. Propaganda and personal bias might be indicated

Analysing a graph – this process can be split into six phases:

1. considering the context and purpose of the graph
2. recognising the type of presentation and examining the axes
3. looking closely at the scale on each axis
4. examining the data presented (e.g. data points, symbols, curves)
5. considering errors and statistics associated with the graph
6. reaching conclusions based on the above.

These processes are amplified in Chapter 74.

Analysing a table – as with analysing a graph, this process can be split into six phases:

1. considering the context and purpose
2. examining the subheadings to see what information is contained in the rows and columns
3. considering the units used and checking any footnotes
4. comparing the data values across rows and/or down columns, looking for patterns, trends and unusual values
5. taking into account any statistics presented
6. reaching conclusions based on the above.

These processes are amplified in more detail in Chapter 73.

by absolute terms, such as ‘everyone knows. . .’; ‘It can be guaranteed that . . .’, or a seemingly one-sided consideration of the evidence. How carefully has the author considered the topic? A less considered approach might be indicated by exaggeration, ambiguity, or the use of ‘journalese’ and slang. Always remember, however, that content should be judged above presentation.

- **Look closely at any numbers quoted** – if the information you are looking at is numerical in form, have statistical errors been taken into consideration, and, where appropriate, quantified? If so, does this help you arrive at a conclusion about how genuine the differences are between important values?
- **Think carefully about any hypothesis-testing statistics used** – are the methods appropriate? Are the underlying hypotheses the right ones? Have the results of any tests been interpreted correctly in arriving at the conclusion? To deal with these matters, you will need at least a basic understanding of the ‘statistical approach’ and of commonly used techniques (see Chapters 76 and 77).

Interpreting data

Numerical data

Information presented in public, whether as a written publication or spoken presentation, is rarely in the same form as it was when first obtained. Chapters 27, 43 and 71 deal with processes in which data are recorded, manipulated and transformed, while Chapter 76 describes the standard descriptive statistics used to ‘encapsulate’ large data sets. Sampling (essentially, obtaining representative measurements) is at the heart of many observational and experimental approaches in biomolecular science (see Chapters 30 and 31), and analysis of samples is a key component of hypothesis-testing statistics (Chapter 77). Understanding these topics and carrying out the associated study exercises will help you improve your ability to interpret numerical data.

Graphs

Frequently, understanding and analysis in science depends on your ability to interpret data presented in graphical form. Sometimes, graphs may mislead. This may be unwitting, as in an unconscious effort to favour a ‘pet’ hypothesis of the author. Graphs may be used to ‘sell’ a product, for example, in advertising, or to favour a viewpoint as, perhaps, in politics. Experience in drawing and interpreting graphs (Chapter 74) will help you spot these flawed presentations, and understanding how graphs can be erroneously presented (Box 74.3) will help you avoid the same pitfalls.

Tables

Tables, especially large ones, can appear as a mass of numbers and thus be more daunting at first sight than graphs. In essence, however, most tables are simpler than most graphs. The construction of tables is dealt with in Chapter 73.

Thinking critically

This involves the application of logic and reasoning to a problem, issue or case study. It requires a wide range of skills. Key processes involved

Learning from examples – as your lecturers introduce you to case studies, you will see how biologists have applied critical thinking to understand the nature of cells, organisms and ecosystems. Some of your laboratory sessions may mimic the processes involved – observation, hypothesis, experimental design, data gathering and analysis and formulating a conclusion (see Chapter 29). These skills and approaches can be applied in your course, e.g. when writing about a biological issue or carrying out a research project.

Looking carefully at statistics – it is sometimes said ‘you can prove anything with statistics’. Leaving aside the issue that statistical methods deal with *probability*, not certainty (Chapter 77), it is possible to analyse and present data in such a way that they support one chosen argument or hypothesis rather than another (Chapter 74). Detecting a bias of this kind can be difficult, but the critical thinking skills involved are essential for all scientists.

include: acquiring and processing information; creating appropriate hypotheses and formulating conclusions; and acting on the conclusions towards a specific objective.

KEY POINT Critical thinking needs reliable knowledge, but it requires you to *use* this appropriately to analyse a problem. It can be contrasted with rote learning – where you might memorise facts without an explicit purpose other than building your knowledge base (see Chapter 3).

Critical thinking is particularly important in biology, because the subject deals with complex and dynamic systems. These can be difficult to understand for several reasons:

- **they are often multi-faceted, involving many interactions**
- **it can be difficult to alter one variable in an experiment without producing confounding variables** (see p. 201)
- **many variables may be unmeasured or immeasurable**
- **heterogeneity (variability) is encountered at all scales** from the molecular scale to the ecosystem
- **perturbation of the system can lead to unexpected (‘counter-intuitive’) results.**

As a result, conclusions in biological research are seldom clear-cut. Critical thinking allows you to arrive at the most probable conclusion from the results at hand; however, it also involves acknowledging that other conclusions might be possible. It allows you to weigh up these possibilities and find a working hypothesis or explanation, but also to understand that your conclusions are essentially dynamic and might alter when new facts are known. Hypothesis-testing with statistics (Chapter 77) is an important adjunct to critical thinking because it demands the formulation of simple hypotheses and provides rational reasons for making conclusions.

Recognising fallacies in arguments is an important aspect of critical thinking. Philosophers and logicians recognise different forms of argument and many different fallacies in each form. Damer (2013) provides an overview of this wide-ranging and complex topic.

Explaining your thoughts

The context for your evaluation of the literature and the associated critical thinking will normally be an essay, report or similar piece of academic writing (Chapters 10–15). The skills involved in marshalling and explaining your thoughts are regarded as highly important in research and employment.

Good writing requires good logic, so understanding the rationale behind what you want to write is a prerequisite for creating high-quality text. Creating a plan for your writing (Chapter 11) will help you to both recognise and organise what you want to say.

You may have a very specific remit, as defined in the instruction for the assignment (Chapter 9), or the topic may be open. In either case, your

Examples**Inductive reasoning**

1. The plant species growing in a mining spoil tip (Site A) are different from those in nearby land subject to similar climatic conditions (Site B).
2. The spoil tip contains a metal Z known to be toxic to a range of organisms.
3. When metal Z is added to pots containing plants from both sites, the plants common on Site B but not on Site A die, but those from Site A all survive.
4. High concentrations of metal Z in the spoil have resulted in the change in species mix due to differential sensitivity to the metal.

Possible flaw in logic: the concentration of metal Z used at step 3 might have been much higher than that found in the spoil and another potentially toxic metal that is present might not have been tested.

Deductive reasoning

1. A bacterial cell has no membrane-bound nucleus.
2. This cell has no membrane-bound nucleus.
3. Therefore this cell is a bacterium.

Possible flaw in logic: other species of birds may also have green backs and no throat markings.

reading around the topic should result in an overarching position or argument you wish to put forward – the ‘thesis’ for your writing. You may be explaining an established viewpoint or creating an original perspective on a topic. For both situations, the same principles apply. When setting out your thoughts, you should:

- **make a clear statement of the issue being considered**, if necessary defining terms and boundaries
- **consider the issue from different perspectives**, providing evidence for or against different propositions
- **cite sources of evidence and ideas** that you are evaluating (Chapter 5)
- **ensure your viewpoint is logical and internally consistent**
- **structure your writing appropriately**, for example, by first considering evidence for a particular view and then evidence against it; or by considering the development of evidence through time
- **use an academic style of writing**, avoiding personal statements (Box 12.2)
- **arrive at a conclusion**, even if this is that the evidence is not sufficient to allow firm statements to be made.

In the sciences, the norm is to use inductive reasoning – that is, to state observed facts and assumptions at the outset, then draw a logical conclusion based on these. The alternative, deductive reasoning, starts from a general statement, premise or law which is held to be true and then reaches a conclusion by considering facts logically. You should look for these types of argument in texts and papers, and also think about possible flaws in such arguments. Chapter 29 discusses further the use of hypotheses and experiments in biological science.

Text reference

Damer, T.E. (2013) *Attacking Faulty Reasoning: A Practical Guide to Fallacy-Free Arguments*, 7th edn. Wadsworth, Belmont, California.

Sources for further study

Anon. *Critical Thinking*. Available: http://en.wikipedia.org/wiki/Critical_thinking
Last accessed 27/04/21.

[A frequently updated review of critical thinking terminology and techniques, with additional links and references.]

Barnard, C.J., Gilbert, F.S. and MacGregor, P.K. (2017) *Asking Questions in Biology: A Guide to Hypothesis Testing, Experimental Design and Presentation in Practical Work and Research Projects*, 5th edn. Pearson Education Ltd, Harlow.

Smith, A. *Evaluation of Information Sources*. Available: http://departments.kings.edu/edtutorial/web_%20evaluation/evalinfosources.htm
Last accessed 27/04/21.

[Part of the Information Quality WWW Virtual Library, containing pointers to a wide range of sources giving criteria for evaluation.]

Van Gelder, T. *Critical Thinking on the Web*. Available: <http://austhink.com/critical/>
Last accessed 27/04/21.

[A useful directory of Web resources on the topic of critical thinking, associated with *Austhink*, a group based in Melbourne, Australia.]

STUDY EXERCISES

6.1 Distinguish between primary and secondary literature.

Based on their titles and any research you can do in your library, determine whether the following journals are primary or secondary sources.

- (a) *British Journal of Haematology*
- (b) *Proceedings of the National Academy of Sciences*
- (c) *Annual Review of Molecular Biology*
- (d) *Essays in Biochemistry*
- (e) *Microbiological Research*
- (f) *Trends in Biotechnology*
- (g) *The Lancet*
- (h) *Oecologia*
- (i) *Scientific American*
- (j) *Food Safety News*

6.2 Consider a controversial issue from both sides.

Select a current biological topic being discussed in the

newspapers or other media. Relatively controversial issues such as 'genetically modified crops' or 'stem cell research' would be good examples. Next, write out a statement that you might use for a motion in a debate, such as 'Genetic modification of crops is a good thing' or 'Creating chimeric organisms is justified if it results in medical advances'. The exercise consists of writing at least five points in support of either side of the argument, which you should organise in tabular form. If you can find more than five points, add these to your table, but for each point that you add to one side, you should add one to the other side.

6.3 Analyse graphic presentations in the media.

Many newspapers provide graphic presentations related to current issues, and graphs are frequently used in television news reports. Practise critical thinking skills by determining whether the graphs presented are a fair representation of the facts.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

7 Working with others

Definitions

Team – a group of people, each of whom has a role within a shared task that is understood by the other members. Members of a team may seek out certain roles and they may perform most effectively in the ones that are most natural to them.

Team role – a tendency to behave, contribute and interrelate with others in a particular way.

(Both after Belbin, 2010)

Gaining confidence through experience – the more you take part in teamwork, the more you know how teams operate and how to make teamwork effective for you.

Taking part in peer assessment – this term applies to marking schemes in which all or a proportion of the marks for a teamwork exercise are allocated by the team members themselves. Read all instructions carefully before embarking on the exercise, so you know which aspects of your work your fellow team members will be assessing. When deciding what marks to allocate to others, try to be fair and balanced in your marking.

It is highly likely that you will be expected to work with fellow students during practicals, tutorials and special study exercises. This might take the form of sharing tasks or casual collaboration through discussion, or it might be through formally directed teamwork, such as problem-based learning (Chapter 3) or organising a poster display (Chapter 15). Interacting with others in this way can be extremely rewarding and realistically represents the professional world, where teamworking is common. Advantages of such collaborative work include:

- **synergistic interactions among team members** – these often result in better ideas, produced by the interchange of views, and better output, due to the complementary skills of team members
- **support offered to individuals by other members of the team**
- **enhanced levels of personal commitment** – because individuals will not want to let the team down
- **sharing responsibilities for complex or difficult tasks**
- **learning from the different approaches of others.**

However, sometimes students are apprehensive about approaching teamwork. Reasons for this include:

- **Initial reservations about working with strangers** – to counter these feelings, it is best to approach group work in terms of positive participation, aiming to establish friendly and productive relationships with others in the team.
- **Concerns about your commitment and experience** – instead, you should view the task as an opportunity to learn more about the topic, and a chance to develop your skills and abilities.
- **Apprehension that the process may be unfair** – if you collectively take care over allocation and monitoring of workloads, this will help keep things in balance, while peer assessment enables you to provide fair marks to other team members. In most cases, everyone will have a common desire to do well, enabling the team to achieve its target.

Teamworking skills

Some of the key skills you will need to develop to optimise your teamworking activities include:

- **interpersonal skills** – these include the way in which you react to new people, how you listen to and communicate with others, and how you deal with conflicts and disagreements
- **delegation and sharing of tasks** – this implies that you are willing to share effort and responsibility, are willing to trust your fellow team members, and have mechanisms to deal with unexpected outcomes or even failure to contribute
- **effective listening** – this involves giving others the opportunity and time to express their views and to take these seriously, however expressed

- **speaking clearly and concisely** – effective communication is a vital part of teamwork, not only between team members, but also when presenting team outcomes to others (see Chapter 16)
- **providing constructive criticism**. It is all too easy to be negative, but only constructive criticism will have a positive effect on interactions with others.

Teamworking dynamics

It is important that team activities are properly structured so that each member knows what is expected of them. Several studies of groups have identified different team roles that derive from differences in personality. Being aware of such categorisations, both in terms of your own predispositions and those of your fellow team members, will help the group to interact more productively. R. M. Belbin identified nine such roles, as shown in Table 7.1.

Allocation of responsibilities usually requires the clear identification of a leader. If asked to form a team, bear the different roles in mind during your selection of colleagues and your interactions with them. The ideal team should contain members who are able to work in all of these roles. However, you should also note the following points:

- **people will probably fit one or more of these roles naturally** as a function of their personality and skills
- **in some circumstances, team members may be required to adapt** and take a different role from the one that they feel suits them
- **people may have to adopt multiple roles**, especially if the team size is small
- **no one role is ‘better’ than any other** – for good teamwork, the group should have a balance of personality types present.

In formal team situations, your module tutor should help the team deal with these issues; even if they do not, it is important that you are aware of these roles and their potential impact on the success or failure of teamwork. You should try to identify your own ‘natural’ role.

KEY POINT In assessed teamwork situations, be clear as to how individual contributions are to be decided. This might require discussion with the course organiser. Make sure that reward in terms of assessment marks, is truly reflective of effort. Failure to ensure that this is fair can lead to disputes and feelings of unfairness.

Working with your lab partner

Many laboratory sessions in the biosciences involve working in pairs. In some cases, you may work with the same partner for a series of practicals or for a complete module. The relationship you develop as a partnership is important to your progress, and can enhance your understanding of

Using Internet-based resources and support for teamwork – websites such as www.belbin.com give further information and practical advice for teamworking.

Noting group discussions – make sure you structure meetings (including creating agendas) and document their outcomes (taking minutes and noting action points).

the practical exercises and, consequently, the grades you obtain. Tips for building a constructive working relationship include:

- **introduce yourselves at the first session** and take a continuing interest in each other's interests and progress at university
- **at appropriate points, discuss the practical** (both theory and tasks) and your understanding of what is expected of you
- **work jointly to complete the practical effectively**, avoiding the situation where one partner dominates the activities and gains most from the practical experience
- **share tasks according to your strengths**, but do this in such a way that one partner can learn new skills and knowledge from the other
- **make sure you ask questions of each other** and communicate any doubts about what you have to do
- **discuss other aspects of your course**, for example, by comparing notes from lectures or ideas about in-course assessments
- **consider meeting up outside the practical sessions** to study, revise and discuss exams.

Table 7.1 How to make the most of the team roles described by Belbin (2010). By understanding the nature of each role, you and your fellow team members can improve your collective performance. No one role should be considered 'better' than any other, and a good team requires members who are able to undertake appropriate roles at different times. Each role provides important strengths to a team, and its compensatory weaknesses should be accepted within the group framework

Team role	Personality characteristics	Typical function in a team	Strengths	Allowable weaknesses
Coordinator	Self-confident, calm and controlled	Leading: causing others to work towards goals	Good at spotting others' talents and delegating activities	Often less creative or intellectual than others in the group
Shaper	Strong need for achievement; outgoing; dynamic; highly strung	Leading: generating action within a team; imposing shape and pattern to work	Providing drive and realism to group activities	Can be headstrong, emotional and less patient than others
Innovator*	Individualistic, serious-minded; often unorthodox	Generating new proposals and solving problems	Creative, innovative and knowledgeable	Tendency to work in isolation; ideas may not always be practical
Monitor-evaluator	Sober, unemotional and prudent	Analysing problems and evaluating ideas	Shrewd judgement	May work slowly; not usually a good motivator
Implementer	Well-organised and self-disciplined, with practical common sense	Doing what needs to be done	Organising abilities and common sense	Lack of flexibility and tendency to resist new ideas
Teamworker	Sociable, mild and sensitive	Being supportive, perceptive and diplomatic; keeping the team going	Good listeners; reliable and flexible; promote team spirit	Not comfortable when leading; may be indecisive
Resource investigator	Extrovert, enthusiastic, curious and communicative	Exploiting opportunities; finding resources; external relations	Quick thinking; good at developing others' ideas	May lose interest rapidly
Completer-finisher	Introvert and anxious; painstaking, orderly and conscientious	Ensuring completion of activity to high standard	Good focus on fulfilling objectives and goals	Obsessive about details; may wish to do all the work to control quality
Specialist	Professional, self-motivated and dedicated	Providing essential skills	Commitment and technical knowledge	Contribute on a narrow aspect of project; tend to be single-minded

* May also be called 'plant' in some texts.

Finding a 'study buddy' – if you feel you might like to team up with someone for study, but lack a suitable partner, tutorial meetings are an opportunity to suggest to others that you might set up further informal meetings. Alternatively, you might consider posting a request on the VLE discussion forum.

Participating in tutorial groups and collaborative study

The group learning environment in tutorials facilitates discussions with others that can help you develop your learning by observing different approaches, views and ideas. This requires all participants to engage fully, while at the same time respecting the contributions and opinions of others. Such learning collaborations can also be less formal – students getting together to exchange materials and ideas in a 'brainstorming' session for a topic or piece of work, or sharing efforts to research a topic. This can be especially valuable when revising (Chapter 17), for example, when:

- **comparing notes, textbooks and other information**
- **going through past papers together, dissecting the questions and planning answers**
- **talking to each other about a topic**
- **explaining difficult parts of the course to each other.**

The synergism and morale boost from working together can be very useful in learning, but where assessment will be based on individual submissions, it is vital that this collaborative learning is distinguished from the collaborative writing of assessed documents. This is not usually acceptable and, in its most extreme form, is plagiarism (p. 41), usually with a heavy punishment. Make sure you know what plagiarism is, what unacceptable collaboration is, and how they are treated within your institution (see Chapter 5).

KEY POINT Collaboration is encouraged during research and learning activities but the final write-up must normally be your own work unless you have specifically been directed to produce a group report.

Using technology for teamwork

Communication is important for effective teamwork, and modern technology can greatly assist this. For example, you can:

- **Send group messages and chat online** – email is the longest-running Internet-based method, but many apps allow near-instant exchange of messages and images (for example, a snapshot of a reference, or a link to a website or online video). Your VLE will probably include discussion forums. Other online topic-based chatrooms may also be useful, allowing you to post questions through online threads and tap into expertise across the globe.
- **Hold virtual meetings** – video conferencing allows group members to discuss matters in real time, avoiding the need for travel or finding a venue. With these, the group should agree on an organiser who is tasked with setting up the meeting and sending messages to confirm the timing. Ideally, someone will act as a chair for the meeting and invite people to contribute by name, as this can avoid the risk of inefficient cross-talking. In terms of noting agreed tasks, responsibilities and timings, the same principles apply as for face-to-face meetings.

Examples of applications for group work

Group messages and chat: WhatsApp®, Instagram® and most email systems

Virtual meetings: Zoom®, BlueJeans®, FaceTime®, Google Meet® and WhatsApp®

Sharing files: Dropbox®, MicroSoft OneDrive®, Google docs®, Google Drive®

Project management: Monday.com®, Wimi®

Holistic systems: Microsoft Teams®, your university's VLE

Evaluating the pros and cons of online teamwork –

Advantages include:

- no need for all group members to be in same location
- improved communication (faster, and potentially including images)
- file sharing with co-writing and editing
- multiple devices supported
- better project management
- improved backup.

Disadvantages include:

- possibility of being diverted from task in hand by chatting rather than working
- in some cases, a need for a version control and file naming strategy
- the requirement for a functioning Internet or Wi-Fi connection
- the costs of necessary hardware and software
- the time taken to source, upload and master the technology.

- **Share and exchange files via cloud computing** – many systems enable files to be stored ‘in the cloud’. These online storage systems can be set up to allow access for all team members, from any location, and on a range of devices from smartphones to personal computers. Using cloud file-sharing avoids the problems of version control (only one copy exists at each editing iteration) and file backup. Wasteful parallel work can be minimised, and there is no need to collate work from different contributors, avoiding potential issues arising from formatting, program or program version differences. Some products incorporate collaboration software, allowing real-time document co-writing and editing.
- **Use project management software** – these applications can help the team coordinate and report on their activity. Many are relatively complex commercial products, so there may be costs involved – both financial and in terms of all group members learning the programme. For many student teamwork projects, these may be too expensive.

Technology-enabled teamworking is a rapidly changing area, so your group should take the recommendations of the lecturer or ask their advice before adopting a specific program or app. Staff may require you to use features on the VLE for groupwork, as this means they can keep track of who has contributed and who hasn't. Technology can also assist in producing a high-quality outcome for that task that has been set, for example, when piecing together individual parts of a team poster for later professional printing at A1 or A0 size (Chapter 15).

Text reference

Belbin, R.M. (2010) *Team Roles at Work*, 2nd edn. Butterworth-Heinemann, Oxford.

Source for further study

Belbin, R.M. *Belbin® Team Roles*.

Available: <https://www.belbin.com/about/belbin-team-roles/>
Last accessed 27/04/21.

STUDY EXERCISES

7.1 Evaluate your ‘natural’ team role(s). Using Table 7.1 as a source, decide which team role best fits your personality. You may wish to consult with a friend or family member for a ‘second opinion’ on your personality traits.

7.2 Keep a journal during a group activity. Record your feelings and observations about experiences of working with other students. After the event, review the journal,

then draw up a strategy for developing aspects where you feel you might have done better.

7.3 Reflect upon your teamwork abilities. Draw up a list of your reactions to previous efforts at collaboration or teamwork and analyse your strengths and weaknesses. How could these interactions have been improved or supported more effectively?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

8 Mapping your personal development

Definitions

Curriculum vitae (CV) – a Latin phrase that means ‘the course your life has taken’. In other words, a record of your achievements.

Personal development plan (PDP) – the record of a process of reflection on personal and career aims (personal development planning). For students, this normally has a focus on outcomes from study and extra-curricular activities. The structure of the plan may be defined by your university, school or department.

During your degree studies, you will be acquiring and refining skills, as well as making progress in terms of the modules you pass. You will also experience personal development from taking part in extracurricular activities. These processes are informed by an underlying plan implicit in both the learning objectives of your curriculum and in the personal decisions you will have made. To ensure your choices are well informed and that your decision-making is in agreement with your overall aims and objectives (Chapter 2), many universities support the creation and maintenance of personal development plans. These provide you with an opportunity to reflect on your aims and your progress towards them. One of the key ways to focus your thoughts in this area is to think forwards to the type of career you might have in mind, and work back to the graduate attributes you would like to develop and the curriculum vitae (CV) you would like to offer to a potential employer. This mapping process will help you not only to refine your ambitions, but also to log your progress in a meaningful and useful way.

KEY POINT Developing your skills and qualities needs to be treated as a long-term project. It makes sense to think early about your career aspirations so that you can make the most of opportunities to build up relevant experience. A good focus for such thoughts is your developing curriculum vitae, so it is useful to work on this from a very early stage.

Identifying different types of PDP portfolio and their benefits – some personal development planning schemes are centred on academic and learning skills, while others are more focused on career planning. Plans or portfolios may be created independently or possibly in tandem with a personal tutor or advisory system. Some schemes operate online, while others are primarily paper based. Each method has specific goals and advantages, but whichever way your scheme operates, maximum benefit will be gained from fully involving yourself in the process.

Personal development planning

If your university has a formal system to support personal development, you will usually be expected to create a portfolio of evidence on your progress, then reflect on this, and subsequently set yourself aims and objectives for the future, including specific targets and action points. The record of your thoughts may be called a personal development plan (PDP), or go under a slightly different name such as a progress file, personal development portfolio or professional development plan. Analysis of your transferable skills profile will probably form part of your PDP (Box 8.1). Other aspects commonly included are:

- **analysis of your aspirations, goals, interests and motivations**
- **your assessment transcript or academic profile information** (e.g. record of grades in your modules)
- **your developing CV** (see p. 56).

Taking part in personal development planning helps you to focus your thoughts about your university studies and future career. This is important in the biomolecular sciences, because most biological sciences degrees can lead to a broad range of occupations. The creation of your PDP will introduce you to some new terms and will help you to describe your personality and abilities. This will be useful when constructing your CV and when applying for jobs.

Box 8.1 How to carry out a personal skills audit

- 1. Create a list of appropriate skills.** If your university publishes a specific skill set, e.g. as part of its framework for PDP, then you should use that. If not, you could adapt the chapter listing on pp. v–vii, or consult a text such as McMillan (2021). Your list should relate to you, your intended career and any specific skills associated with your intended qualification.
- 2. Lay out your list in table format.** This should have four columns, as shown in Table 8.1.
- 3. Rate your skills.** This may be challenging for many students as it is difficult to be objective and tough to gauge employer expectations. If you feel confident, you may rate a certain skill strongly, while a self-critical person may consider the same level of skill to be deficient. However, this does not matter too much as you will effectively be comparing yourself at different stages in your learning, rather than judging yourself against an outside standard. One method is to use a scale of 1 to 10, with low values indicating that the skill ‘needs lots of development’ and high values indicating that, for the time being, you feel your competence is ‘well above average’.
- 4. Note actions.** This especially applies to skills with low scores in the previous column – and you may wish to prioritise certain ones. You will need to think about ways in which you could improve, and this may require some research on your part. Is there a book or website you could read? Is there a training workshop you could attend or online video you could view? Could an extracurricular activity help you to develop your skills? Should you sign up to speak to a skills adviser? It is important that you recognise that the solution to any deficiencies you perceive lies in your own hands. At university, no one will do the work for you.
- 5. Add comments and progress notes.** Here is where you can add any comments to amplify or assist with the action points. The addition of progress notes implies that you will revisit the list from time to time. If your university system for PDPs allows you to add the list to a portfolio, then do this.

Inevitably, your skills audit will become out of date after a period. It will still be useful, however, to look back at so you can see how you have progressed. This will give a sense of achievement and self-awareness that could be valuable when speaking to academic tutors or careers advisers and potential employers. You may wish to set up a new list at intervals, perhaps at the start of each academic year.

Table 8.1 One possible way of creating a personal skills audit. The second row provides guidance about the content of each column. The third row provides an example of possible content

Skill	Rating at [date] with notes	Proposed actions	Comments and notes on progress
You should be quite specific. It may be a good idea to subdivide complex skills like ‘communication’	Provide a realistic evaluation of your competence in the skill at a specific point in time	Note what you intend to do to try to improve the skill. You might tick these off as completed	Summarise your progress. You may wish to add a revised rating
Giving spoken presentations	4/10 [3rd March 2021] Wasn't satisfied with presentation to tutorial group – nervous, a little disorganised and my PowerPoint slides were too ‘wordy’	1. Read Ch 11 in Practical Skills in Biomolecular Sciences ✓ 2. Learn how to use advanced features of PowerPoint ✓ 3. Ask more questions in tutorials ✓	Gave second presentation to tutorial group; went well, although quite nervous at start. Slides much better. Make sure not to rush the introduction next time. 7/10

Evaluating your abilities – it may be helpful to pause to think about the skills and personal qualities you are developing in both your studies and your extracurricular activities, as this will allow you to appreciate better what you are doing and why.

Assessing your skills and personal qualities

Skills (sometimes called competences) are generally what you have learned to do and have improved with practice. Regarding academic, intellectual and practical skills, your tutors will have designed your courses to give you plenty of opportunities for developing your expertise, such as those highlighted in Table 8.2. Personal qualities, on the other hand, are predominately innate. Examples include honesty, determination and thoroughness (Table 8.3). These qualities need not remain static, however, and can be developed or changed according to your experiences.

Table 8.2 Skills related to the biomolecular sciences. A selection of general skills likely to be developed during your degree

Analysing and evaluating information
Critical thinking
Designing experiments and surveys
Field work
Hypothesis testing
Interpreting data
IT skills
Lab work
Numeracy
Organisational skills
Presentational skills
Presenting data
Problem solving
Processing data
Reporting results
Research skills
Teamwork
Time management

Table 8.3 Some positive personal qualities

Adaptability
Conscientiousness
Curiosity
Determination
Drive
Energy
Enthusiasm
Fitness and health
Flexible approach
Honesty
Innovation
Integrity
Leadership
Logical approach
Motivation
Patience
Performance under stress
Perseverance
Prudence
Quickness of thought
Seeing others' viewpoints
Self-confidence
Self-discipline
Sense of purpose
Shrewd judgement
Social skills (sociability)
Taking initiative
Tenacity
Tidiness
Thoroughness
Tolerance
Unemotional approach
Willingness to take on challenges

Personal qualities and skills are interrelated because your personal qualities can influence the skills you gain. For example, you may become highly proficient at a skill requiring manual dexterity if you are particularly adept with your hands. Being able to transfer your skills is highly important (Chapter 1) – many employers take a long-term view and look for evidence of the adaptability that will allow you to be a flexible employee and one who will continue to develop skills.

Assessing your skills may be easier than critically analysing your personal characteristics. In judging your qualities, try to take a positive view and avoid being overly modest. It is important to consider your qualities in a specific context, for example, 'I have shown that I am trustworthy by acting as treasurer for the Biochemical Society', as this evidence will form a vital part of your CV and job applications.

Developing your curriculum vitae

While some students only think about their curriculum vitae immediately before applying for a job, the planning, developing and revising of your CV should be a long-term project. There are four main reasons why this mapping process can be valuable:

- 1. Evaluating your CV and how it will look to a future employer will help you think more deeply about the direction and value of your academic studies.**
- 2. Creating a draft CV will prompt you to assess your skills and personal qualities** and, perhaps in conjunction with your personal development plan, how these fit into your career aspirations.
- 3. Your CV can be used as a record of all the relevant things you have done at university** and then, later, when modified suitably, will help you communicate these to a potential employer.
- 4. Your developing CV can be used when you apply for vacation or part-time employment.**

If you can identify gaps in your skills profile, or qualities that you would like to develop, especially in relation to the needs of your intended career, the next step is to think about ways of improving them. This will be reasonably easy in some cases, but may require some creative thinking in others. A relatively simple example would be if you decided to learn a new language or to sustain one you learned at school. There are likely to be many local college and university courses dealing with foreign languages at many different levels, so it would be a straightforward matter to join one of these. A rather more difficult case might be if you wished to demonstrate 'responsibility', because there are no courses available on this. One route to demonstrate this quality might be to put yourself up for election as an officer in a student society or club; another could be to take a leading role in a relevant activity within your community (for example, voluntary work such as hospital radio). If you already take part in activities like these, your CV should relate them to this context.

Thinking about a future career

You may find it difficult to plan ahead or evaluate your personal development needs without a specific career in mind – and many choose biomolecular science as a subject precisely because it is a pathway to a wide variety of occupations. Many of these will directly use your knowledge and skills

Focusing on evidence – it is important to be able to provide specific information that will back up the claims you make under the ‘skills and personal qualities’ and other sections of your CV. A potential employer will be interested in your level of competence (what you can actually do) and in situations where you have used a skill or demonstrated a particular quality. These aspects can also be mentioned in your covering letter or at interview.

Examples Main areas of degree-related employment for biosciences graduates, according to the UK QAA (QAA, 2019):

- bioscience, biotechnology and healthcare industries
- commerce (sales and marketing) related to healthcare, agricultural and diagnostic products
- diagnostic laboratories
- education: university, college and school teaching
- environmental and conservation projects
- food industry and food safety
- forensic laboratories
- government departments and the civil service
- government or charity-funded research laboratories and institutes
- international organisations
- patent offices
- pathology laboratories
- regulatory matters in healthcare, including clinical trials
- research and development for agri-environment industries
- research laboratories in universities
- science communication and public engagement
- science policy
- veterinary and agricultural laboratories.

in the biosciences, but others will favour the general intellectual skills you develop during your degree. However, thinking about your future can help you become clearer about your ambitions and refine your aims (Chapter 2). If you feel you need assistance you should discuss your situation with Careers Service staff, who will not only have wide experience and depth of knowledge about potential careers, but access to valuable information sources.

Structuring and presenting your curriculum vitae

Box 8.2 illustrates the typical parts of a CV and explains the purpose of each part. Employers are more likely to take notice of a CV that is well organised and presented, in contrast to one that is difficult to read. They will expect it to be concise, complete and accurate. There are many ways of presenting information in a CV, and you will be assessed partly on your choices.

- **Order.** There is some flexibility as to the order in which you can present the different parts (see Box 8.2). A chronological approach within sections helps employers gain a picture of your experience.
- **Personality and ‘colour’.** Make your CV different by avoiding standard or dull phrasing. Try not to focus solely on academic aspects: you will probably have to work in a team and the social aspects of teamwork will be enhanced by your outside interests. However, make sure that the reader does not get the impression that these interests dominate your life.
- **Style.** Your CV should reflect *your* personality, but not in such a way that it indicates too idiosyncratic an approach. It is probably better to be formal in both language and presentation, as flippant or chatty expressions will not be well received.
- **Neatness.** Producing a well-presented, word-processed CV is very important. Use a laser-quality printer and good-quality paper; avoid poor-quality photocopying at all costs.
- **Layout.** Use headings for different aspects, such as personal details, education, etc. Emphasise words (for example, with capitals, bold, italics or underlining) sparingly and with the primary aim of making the structure clearer. Remember that careful use of white space is important in design.
- **Grammar and proofreading.** Look at your CV carefully before you submit it, as sloppy errors give a very poor impression. Even if you use a spellchecker, some errors may creep in. Ask someone whom you regard as a reliable proofreader to comment on it (many tutors will do this, if asked in advance).
- **Relevance.** If you can, slant your CV towards the job description and the qualifications required (see below). Make sure you provide evidence to back up your assertions about skills, qualities and experience.
- **Accuracy and completeness.** Check that all your dates tally; otherwise, you will seem careless. It is better to be honest about your grades and, for example, a period of unemployment, than to cover this up or omit details that an employer will want to know. They may be suspicious if you leave things out.

Seeing yourself as others see you – you may not recognise all of your personal qualities and you may need someone else to give you a frank appraisal. This could be anyone whose opinion you value: a friend, a member of your family, a tutor, or a careers adviser.

Setting your own agenda – you have the capability to widen your experience and to demonstrate relevant personal qualities through both curricular and extra-curricular activities.

Paying attention to the quality of your CV – your potential employer will regard your CV as an example of your very best work and will not be impressed if it is full of mistakes or badly presented, especially if you claim ‘good written communication’ as a skill!

Tailoring your CV to suit your application

You should fine-tune your CV for each submission. Highlight relevant qualifications as early in your CV as possible. Be selective – do not include every detail about yourself. Emphasise relevant parts and leave out irrelevant details, according to the job. Employers frequently use a ‘person specification’ to define the skills and qualities demanded in a job, often under headings such as ‘essential’ and ‘desirable’. This will help you decide whether to apply for a position and it assists the selection panel to filter the applicants.

Increasingly, applicants are asked to write a document that specifically addresses the person specification and associated criteria, so make sure what you write matches your CV. Remember that your letter of application is not merely a formal document but is also an opportunity for persuasion (Box 8.2). You can use it to state your ambitions and highlight particular qualifications and experience. However, don’t go over the top – always keep the letter to a single page.

As you may apply for several jobs, it is useful to construct a CV which includes *all* information of potential relevance. This can then be modified to fit each post. Having a prepared CV on file will reduce the work each time you apply, while modifying this will help you focus on relevant skills and attributes for the particular job.

KEY POINT A well-constructed and relevant CV will not necessarily guarantee you a job, but it may well get you on to the short list for interview. A poor-quality CV is a sure route to failure.

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Box 8.2 How to structure a CV and covering letter

There is no right or wrong way to write a CV, and no single format applies. It is probably best to avoid software templates and CV 'wizards' as they can create a bland, standardised result, rather than something that demonstrates your individuality.

You should include the following with appropriate subheadings, generally in the order given below.

- 1. Personal details.** This section *must* include your full name and date of birth, your address (both home and term-time, with dates, if appropriate) and a contact telephone number at each address. If you have an email account, you might also include this.
- 2. Education.** Choose either chronological order, or reverse chronological order and make sure you take the same approach in all other sections. Give educational institutions and dates (month, year) and provide more detail for your degree course than for your previous education. Remember to mention any prizes, scholarships or other academic achievements. Include your overall mark for the most recent year of your course, if it seems appropriate. Make sure you explain any gap years.
- 3. Work experience.** Include all temporary, part-time, full-time or voluntary jobs. Details include dates, employer, job title and major duties involved.
- 4. Skills and personal qualities.** Emphasise your strengths, and tailor this section to the specific requirements of the post (the 'job description'): for example, you might emphasise the practical skills you have gained during your degree studies if the post is a biological one, but concentrate on generic transferable skills and personal qualities for other jobs. Provide supporting evidence for your statements in all cases.
- 5. Interests and activities.** This is an opportunity to bring out the positive aspects of your personality, and explain their relevance to the post you are applying for. Aim to keep this section brief, or it may seem to the reader that your social life is more important than your education and work experience. Include up to four separate items, and provide sufficient detail to highlight the *positive* aspects of your interests (e.g. positions of responsibility, working with others, communication, etc.). Use Sections 4 and 5 to demonstrate that you have the necessary attributes to fulfil the major requirements of the post.
- 6. Referees.** Include the names (and titles), job descriptions, full postal addresses, contact telephone numbers and email addresses of two referees (rarely, some employers may ask for three). It is usual to include your personal tutor or course leader at university (who among other things

will verify your marks), plus another person – perhaps a current or former employer, or someone who runs a club or society and who knows your personal interests and activities. Unless you have kept in touch with a particular teacher since starting university, it is probably best to choose current contacts, rather than those from your previous education.

Some other points to consider:

- **Try to avoid jargon and over-complicated phrases in your CV** – aim for direct, active words and phrases (see Box 16.1).
- **Employers will expect your CV to be well written** – errors in style, grammar and presentation will count against you, so be sure to check through your final version (and ask a reliable person to second-check it for you).
- **Aim for a maximum length of two pages** – your CV should be printed single-sided on A4 paper, using a 'formal' font (e.g. Times Roman or Arial) of no less than 12 point for the main text. Always print on good-quality white paper. Avoid fussy use of colour, borders or fonts.
- **Do not try to cram in too much detail** – use a clear and succinct approach with short sentences and lists to improve 'readability' and create structure. Remember that your aim is to catch the eye of your potential employer, who may have many applications to work through.
- **It is polite to check that people are willing to act as a referee for you** – and to provide them with an up-to-date copy of your CV.

Your covering letter should have four major components:

- 1. Letterhead.** Include your contact details, the recipient's name and title (if known) and address, plus any job reference number.
- 2. Introductory paragraph.** Explain who you are and *state the post you are applying for*.
- 3. Main message.** This is your opportunity to sell yourself to a potential employer, highlighting particular attributes and experience. Keep it to three or four sentences at the most and relate it to the particular skills and qualities demanded in the job or person specification.
- 4. Concluding paragraph.** A brief statement that you look forward to hearing the outcome of your application is sufficient.

Finally, add either 'Yours sincerely' (where the recipient's name is known) or 'Yours faithfully' (in a letter beginning 'Dear Sir or Madam') and then end with your signature.

STUDY EXERCISES

8.1 Evaluate your personal attributes. Using Table 8.3 as a source, list *five* qualities that you feel that you would best use to describe yourself, and cite the evidence you might give to a potential employer to convince them that this was the case. List *five* attributes you could develop, and then indicate how you might do this.

8.2 Create a generic CV. Drawing on your school record of achievement or similar, or any CV you may already have prepared, for example, for a summer or part-time job, create a word-processed generic CV. Save the file in

an appropriate (computer) folder and make a back-up copy. Print out a copy for filing. Periodically update the word-processed version. If necessary, keep or save different versions to be used in different contexts (e.g. when you apply for a vacation job).

8.3 Think about your future career and ask for advice.

Make an appointment with one of the advisers in your university's careers service. Ask what the career options might be for people with your intended degree, or determine what qualifications or module options might be appropriate for occupations that attract you.

Answers to these study exercises are available at go.pearson.com/uk/he/resources



Assessment skills

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9 Succeeding in assessments

Understanding the independence of university assessment systems – the essence of being a university is that a governance charter bestows on the institution the right to teach students, to set assessments, provide grades and award degrees. Each university has broadly similar, but subtly different, methods of assessing students, and each subject has its specific methodologies and conventions. These are monitored and controlled through national quality assurance agencies and the external examiner system.

Definitions

Module (unit) – a component element of a programme, often a semester (term) or half-semester long, consisting of teaching on a series of related topics.

Course (Programme) – the specific combination(s) of modules or units at different levels of study required for the award of a named degree.

Transcript – your official record of achievement at university, providing details of the modules you took and the grades you received.

Understanding honours degree classifications – in the UK, these are normally:

- 1st class
- 2:1 or upper second class
- 2:2 or lower second class
- 3rd class
- Pass or ordinary degree.

The average marks or grades required for each classification depend on the institution's rules.

In higher education, you will be expected to navigate your own way around the 'rulebook' for assessments; this is unlike school systems, where your teachers would have provided direct guidance. Consequently, you need to get to grips with your university's assessment system for coursework and exams. Your aim should be to understand what you need to learn, how your learning will be graded and, consequently, how you can best prepare for success in assessment.

KEY POINT Your department or school will provide information in course (programme) and module (unit) handbooks that you can use to plan your approach and maximise your performance in assessment.

Understanding the assessment system at your university

Working from broad information on how degrees are awarded, down to the marking of individual assessments, you need to know and apply the following:

- **How your module marks contribute to your final degree classification.** In the 'grade point average' model, all module marks count (possibly weighted in favour of later years); in the 'finals' model, the marks in 'high stakes' end-of-degree (final year) exams are counted, in some cases using a small proportion of marks obtained in other types of assessment. In the former case, your strategy must be to do as well as possible in all assessments, while the temptation for the latter is to leave 'serious' revision until your last year. That is not a good plan: not only because early, focussed study lays the foundations for future success, but also because you will receive an official 'transcript' at the end of your degree, which outlines all of your achievements. A future employer might ask to see this document, and will be interested in your grades at all levels. Whatever the method used to arrive at your degree classification, you should develop a strategy for your overall approach to assessment while at university.
- **The importance of progression criteria.** As you move between levels of the university system, you will need to pass certain modules to qualify for the next stage, as detailed in your department or school's progression criteria. Students are normally allowed two attempts to pass each module and the resits/reassessments often take place at the end of the summer vacation. If a student does not pass at their second attempt, they may be asked to 'carry' the subject in a subsequent year, and in severe cases of multiple failure, they may be asked to retake the whole year or even leave the course. Importantly, module marks may also be used to decide whether you qualify for specific degree pathways. Consequently, it is worth finding out about these aspects of your course/degree as early as possible – they are usually published in relevant programme and module handbooks and may be expressed using a credit-point system. If you have ambitions for a good degree, your goal should be always to perform at your highest possible level and not simply to fulfil the minimum criteria for progression.

Taking account of competition for degree pathways – the numbers of students able to study for certain degrees is sometimes constrained by resources, and entry to pathways may be decided on the basis of performance in early modules. Staff may also restrict entry to those with proven ability in certain areas, for example, numeracy. You should speak with tutors to find out about such restrictions.

Understanding credits and study levels – in the UK, the volume and difficulty of university teaching are described in broad terms by:

- **Credit points** – one point is allocated per 10 hours of notional teaching (including self-study and assessment). One year of an undergraduate degree consists of 120 credit points, split among the component modules taken.
- **The relevant national qualifications framework** – this outlines the expectations for different stages of study, ranging from school level through undergraduate to postgraduate.

A given module will be rated for its volume in terms of credits and its difficulty in terms of level. You will need to pass an appropriate number of modules at each level of study to gain your degree. Through assumed equivalence, these systems can account for prior learning and can facilitate movement between programmes, educational institutions and different teaching systems. Analogous schemes operate in other countries, enabling international student transfers and periods of overseas study.

- **The educational goals for different types of assessment.** ‘Formative assessments’, typically mid-module tests, tutorial questions and in-class problem-solving exercises, aim to give you feedback on your performance under ‘low stakes’ conditions, with little or no contribution to your final mark. ‘Summative assessments’ include end-of-programme, end-of-year or end-of-module exams and other assessments. The main aim here is for staff to evaluate your learning. In ‘continuous’ or ‘in-course’ assessment, summative elements are spread out over the course. These coursework assessments – often termed ‘assignments’ – may also include a formative aspect, if feedback is given. ‘Peer assessment’ may be involved in some cases. ‘Self-assessment’ is where you grade your own performance, as part of the learning process, typically with no credit towards your module marks (Chapter 3). Self-assessment questions may be provided as part of your learning resources and/or core texts (see examples at the end of each chapter of this book). You should aim to take advantage of all elements of the assessment system by using each experience to learn how best to prepare for, and complete, the different types of future assessments.
- **How each element of assessment counts towards your module grade.** The timing and nature of individual components and their weighting will be covered in the guidance notes for each module. This information should inform the way you spread your effort and carry out each assessment task. Ensuring you submit each assignment on time and prepare well for each exam paper will maximise your chance of a good mark.
- **What staff expect you to understand.** ‘Learning objectives’ (or ‘learning outcomes’) are statements of the knowledge, understanding or skills that a learner should be able to demonstrate on successful completion of a module, topic or learning activity (Chapter 17). Coursework assignments and exam questions are often closely based on these, so they should always be your starting point when preparing for assessment. You will find them in your module guide or handbook. You will probably notice that learning objectives, like marking criteria, can be mapped to the cognition levels described in Table 3.1: essentially, the higher your level of study, the higher the level of cognition expected. To do well, you will need to match your work to your tutors’ expectations.
- **How staff arrive at your grades.** ‘Assessment criteria’ set out the specific aspects of knowledge and skills that will be used to determine a mark/grade for an assessment task while assessment scales show the system used to communicate your grade, often in relation to the degree classifications (for example ‘A2’ for a mid-range first-class answer). The latter are often the same across your department/school/faculty/university, and for that reason you may find them on a central website. If you want to do well, it is important to understand these criteria and scales, as they help to explain the nature of the answers you should be giving to achieve a desired grade.
- **Ratification of grades.** In certain assessments, double- or multiple-markers may assess your work to arrive at an agreed grade and the external examiner may also confirm standards from a sample of scripts.

Example A set of learning outcomes taken from an introductory lecture on bacterial cell structure.

After this lecture, you will be able to:

- Define the following terms:
 - prokaryote
 - eukaryote
 - envelope
 - fimbriae
 - F pilus
 - plasmid.
- Draw a labelled diagram to illustrate the principal components of a bacterial cell.
- Explain the functions of the major cellular components.
- Demonstrate knowledge of the relative magnitude of bacteria and eukaryotic cells, in terms of typical linear dimensions and volumes.
- Describe the basic process of cell division and give examples of typical timescales for different bacteria, e.g. *Escherichia coli*, *Clostridium perfringens*, *Mycobacterium tuberculosis*.

Example These are typical marking criteria for a first-class answer in biology:

- contains all of the information required with either no or only a few minor errors
- shows evidence of having read relevant literature and uses this effectively in the answer
- addresses the question directly, understanding all its nuances
- includes little or no irrelevant material
- demonstrates a full understanding of the topic in a wider context
- shows good critical and analytical abilities
- contains evidence of sound independent thinking
- expresses ideas clearly and concisely
- written logically and with appropriate structure
- standard of English very high
- detailed and relevant diagrams used.

Using assessment information

The following are useful sources:

- **The module handbook (or assessment area of your virtual learning environment)** – the place where you will find information on coursework assignments and exams, and consequently, the nature and timing of the studying you will need to do for them.
- **Past exam papers and model answers** – these can give an idea of the style and format of the questions likely to be set, and the level of answer required. Analysing both of these can help you judge the nature of the study and revision required and the standard you should be aiming towards. Aligning current and past questions to learning objectives/outcomes will help you to understand the relationship between these elements (but check that the objective/outcomes haven't changed).
- **Tutor feedback** – assessment that is primarily geared towards feedback ('assessment for learning'), enables you to learn from (i) the overall mark/grade and, perhaps more importantly, (ii) through written comments and notes on your work. For such assessments, make sure that you understand the basis of your mark/grade and also the comments provided by your tutor – most will be pleased to explain how you can improve your marks.
- **Past performance** – you should reflect on the grades you have achieved in the past, especially in relation to the effort you put into the relevant exercise. Sometimes you can explain your marks easily, for example, a misunderstanding about the question, or a weak exam strategy. This should help you to recognise where you need to allocate more effort in study or revision for future assessments.

Improving your marks

Coursework

In relation to your planning and performance, one benefit of having coursework assignments is that they come as no surprise. From your module handbook, you will know from the outset:

- **the timing of the work**, that is, the submission dates
- **the nature of the work**, for example, the type and number of exercises and
- **how much the work counts towards the overall module mark or grade.**

You can therefore plan periods of time when you can do the necessary work (Chapter 2). This process should take account of the requirements of other modules: one of the benefits of advance planning is avoiding timetable clashes. With this advance information, you can also obtain relevant books and references, or search for the URLs of relevant websites. However, make sure you note down all the relevant citation information for use when the time comes to refer to these sources (Chapter 5).

In theory, it should be easier to gain good marks in coursework if you put in sufficient effort. In 'mixed' assessment schemes, which use both assignments and exams, you should therefore be able to create a sound base to build on in the final exam. Another benefit of coursework is the wide range of activities covered – some of these may play to your strengths.

Examples The common types of course-work assignments (and where they are covered in this book) are:

- data analysis (Chapters 71, 72, 75 and 76)
- essays (Chapters 10 and 11)
- literature surveys and reviews (Chapter 13)
- numerical problems (Chapter 75)
- outcomes from problem-based learning (Chapter 3)
- poster displays (Chapter 15)
- practical exercises and reports (throughout)
- project work (Chapters 12 and 32)
- spoken presentations (Chapter 16).

Thinking about the way your script will be graded – individual markers may produce a ‘marking scheme’ to help them assess scripts – for example, these may allocate marks for:

- including a relevant definition
- adding a well-labelled diagram
- explaining a specific piece of research
- showing the stages of a calculation
- using an appropriate style of writing.

Predicting the potential contents of such schemes can help you to include all the material likely to be assessed. They are likely to be highly correlated with the learning outcomes/objectives for the relevant topic.

Understanding the role of the external examiner – this external appointee, usually an accomplished person in the subject, has the task of adjudicating on and confirming the standards set in marking and awarding degrees. They may also advise on the nature of assessment and even on specific details such as the wording of assessment questions.

Other tips for achieving better coursework grades are to:

- **discuss the task with fellow students at an early stage to ensure you have understood the nuances of the task correctly** – if you can’t agree, consult a tutor
- **consult relevant chapters in this book to help you with the tasks**
- **work with fellow students to find and share relevant source material** – but do not collaborate when writing the final submission, unless it is a group assessment (Chapter 7)
- **ensure you take account of material presented in lectures and practicals** – this may give clues as to the approach you will be expected to take in your coursework
- **in group work, take on the role of ‘organiser’ (Chapter 7) at an early stage, if appropriate** – this will help get the project going
- **start writing up at an early stage** – bear in mind that you do not have to start at the beginning (Chapters 11–13)
- **take full account of feedback** – read the comments on your work as soon as it is returned, so that you can ask for an explanation while the topic is fresh in your mind. If you find that the same comments appear repeatedly, it may be a good idea to seek help from your university’s academic support unit.

KEY POINT Success in coursework requires planning and sustained effort throughout each module. It is particularly important to allocate your time and effort for each assessment in proportion to its contribution to the overall module mark/grade.

Exams

The aim of ‘high stakes’ exams is to provide an indication of your ability to work independently, without the aid of source material or being able to consult other people. The normal resources you would consult for coursework (for example, websites, textbooks, and research papers) are replaced by information stored in your memory. This emphasises the importance of the revision process, so to perform well, you will need a strategic approach for this, as detailed in Chapter 17.

In so-called ‘open book’ exams you may be able to consult specific resources, usually textbooks, but also sometimes notes and the Internet. However, this is not a reason for being unprepared. The questions will probably centre on *how you use* information, rather than simply reporting it. You may wish to add Post-it notes as bookmarks to your textbook to help you find specific material more quickly. Although you will need to know where to find relevant facts quickly, you will also need to demonstrate higher level cognitive skills and critical thinking (Chapter 3).

You will find yourself time-limited during most exams – as well as needing to be able to recall the relevant material, you will need an effective plan for the time allotted. Chapter 18 covers how to create and implement an exam strategy.

KEY POINT Success in exams requires a strategic approach, both to revision and to the exam itself.

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STUDY EXERCISES

9.1 Consult the handbook for an upcoming module.

Find out:

- the types of assessment involved
- the submission and exam dates
- the proportion each assignment/exam counts towards your module grade
- whether any assessments have a minimum mark for progression.

Use this information to create a study plan for the module, accounting for advance reading/research required for assignments and any revision period required for tests and exams.

9.2 Research the marking criteria used in your department/school. Relate this information to marks you have already received for assignments and exams. In combination with any feedback received, do the criteria throw light on the reasons for your grades? How might you address any shortfall between your performance and your ambition?

9.3 Make use of feedback. The purpose of tutor feedback is to help you perform better in subsequent assessments. One strategy to achieve this is to write out each of the major comments from one of your assignments in the first column of a two-column table. Then, in the second column, summarise how you intend to address each comment/aspect.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

10 Scientific writing

Monday:	morning afternoon evening	Lectures (University) Practical (University) Initial analysis and brainstorming (Home)
Tuesday:	morning afternoon evening	Lectures (University) Locate sources (Library) Background reading (Library)
Wednesday:	morning afternoon evening	Background reading (Library) Squash (Sports hall) Planning (Home)
Thursday:	morning afternoon evening	Lectures (University) Additional reading (Library) Prepare outline (Library)
Friday:	morning afternoon evening	Lab class (University) Write first draft (Home) Complete first draft, go out with friends (When finished)
Saturday:	morning afternoon evening	Shopping (Town) Review first draft (Home) Revise first draft (Home)
Sunday:	morning afternoon evening	Free Produce final copy (Home) Proofread then print essay (Home)
Monday:	morning	Final read-through and check Submit essay (deadline midday)

Fig. 10.1 Example timetable for writing a short essay.

Written communication is an essential component of all sciences. Most courses include writing exercises in which you will learn to describe ideas and results accurately, succinctly and in an appropriate style and format. The following features are common to all forms of scientific writing.

Organising your time

Making a timetable at the outset helps ensure that you give each stage adequate attention and complete the work on time (for example, Fig. 10.1). To create and use a timetable:

1. Break down the task into stages.
2. Decide on the proportion of the total time each stage should take.
3. Set realistic deadlines for completing each stage, allowing some time for slippage.
4. Refer to your timetable frequently as you work: if you fail to meet one of your deadlines, make a serious effort to catch up as soon as possible, if necessary, by not carrying out a discretionary activity.

KEY POINT The appropriate allocation of your time to reading, planning, writing and revising will differ according to the task in hand (see Chapters 11–13).

Organising your information and ideas

Before you write, you need to gather and/or think about relevant material (Chapters 5 and 6). You must then decide:

- what needs to be included and what does not
- in what order it should appear.

Start by jotting down headings for everything of potential relevance to the topic (this is sometimes called ‘brainstorming’). A spider diagram (Fig. 10.2) or a Mind Map (Fig. 4.2) will help you organise these ideas. The next stage is to create an outline of your text (Fig. 10.3). Outlines are valuable because they:

- force you to think about and plan the structure
- provide a checklist so nothing is missed out
- ensure the material is balanced in content and length
- help you organise figures and tables, by showing where they will be used.

However, you should not spend too long on this phase at the expense of time spend doing the writing itself.

KEY POINT A suitable structure is essential to the narrative of your writing, and should be carefully considered at the outset.

Creating an outline – an informal outline can be made simply by indicating the order of sections on a spider diagram (as shown in Fig. 10.2).

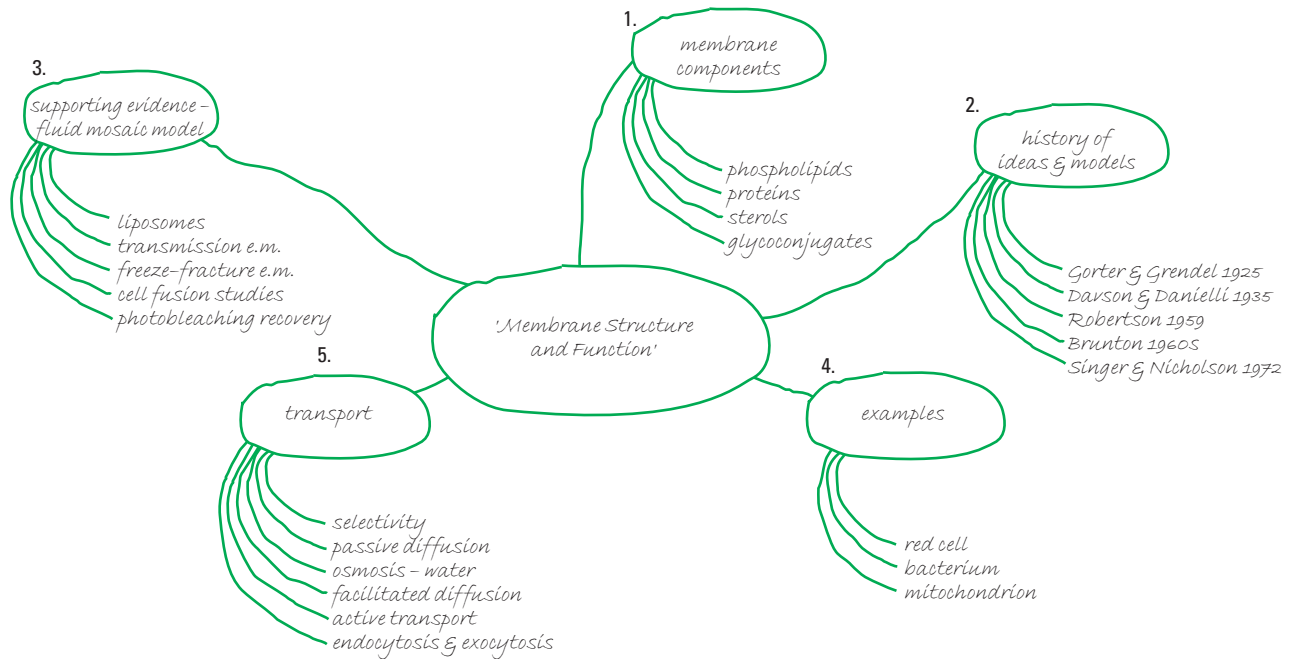


Fig. 10.2 Example of a spider diagram. This shows how you might 'brainstorm' an essay with the title 'Membrane Structure and Function'. Write out the essay title in full to form the spider's body, and as you think of possible content, place headings around this to form the spider's legs. Decide which headings are relevant and which are not, and use arrows to note connections between subjects. This may influence your choice of order and may help to make your writing flow because the links between paragraphs will be natural. You can make an informal outline directly on a spider diagram by adding numbers indicating a sequence of paragraphs (as shown). This method is best when you must work quickly, as with an essay written under exam conditions.

Fig. 10.3 Examples of formal outlines. These are useful for a long piece of work where you or the reader might otherwise lose track of the structure. The headings for sections and paragraphs are simply written in sequence with the type of lettering and level of indentation indicating their hierarchy. Two different forms of formal outline are shown, a minimal form (a) and a numbered form (b). Note that the headings used in an outline are often repeated within the essay to emphasise its structure. The content of an outline will depend on the time you have available and the nature of the work, but the most detailed hierarchy you should reasonably include is the subject of each paragraph.

Membrane structure and function	Membrane structure and function
Introduction	1. INTRODUCTION
membrane components	1.1 membrane components
phospholipids	1.1.1 phospholipids
proteins	1.1.2 proteins
sterols	1.1.3 sterols
glycoconjugates	1.1.4 glycoconjugates
history of ideas & models	1.2 history of ideas & models
Gorter & Grendel 1925	1.2.1 Gorter & Grendel 1925
Davson & Danielli 1935	1.2.2 Davson & Danielli 1935
Robertson 1959	1.2.3 Robertson 1959
Brunton 1960s	1.2.4 Brunton 1960s
Singer & Nicholson 1972	1.2.5 Singer & Nicholson 1972
Supporting evidence – fluid mosaic model	2. SUPPORTING EVIDENCE – FLUID MOSAIC MODEL
liposomes	2.1 liposomes
transmission e.m.	2.2 transmission e.m.
freeze–fracture e.m.	2.3 freeze–fracture e.m.
cell fusion studies	2.4 cell fusion studies
photobleaching recovery	2.5 photobleaching recovery
Transport	3. TRANSPORT
selectivity	3.1 selectivity
passive diffusion	3.2 passive diffusion
osmosis – water	3.3 osmosis – water
facilitated diffusion	3.4 facilitated diffusion
active transport	3.5 active transport
endocytosis & exocytosis	3.6 endocytosis & exocytosis
Examples	4. EXAMPLES
red cell	4.1 red cell
bacterium	4.2 bacterium
mitochondrion	4.3 mitochondrion

(a)

(b)

Benefitting from talking about your work – discussing your topic with a friend or colleague might bring out ideas or reveal deficiencies in your knowledge.

Improving your writing skills – you need to take a long-term view if you wish to improve this aspect of your work. Much of the information you might want (e.g. synonyms) is available online or associated with word processing programs. However, you may wish to create a personal reference library (see Box 10.3).

Making the most of technology – use the dynamic and interactive features of word processing (Chapter 14) to help you get started: first make notes on structure and content, then expand these to form a first draft and finally revise/improve the text.

In an essay or review, the structure of your writing should help the reader to assimilate and understand your main points. Subdivisions of the topic could simply be related to the physical nature of the subject matter (for example, zones of an ecosystem) and should proceed logically (for example, low-water mark to high-water mark). A chronological approach is good for evaluation of past work (for example, the development of the concept of evolution), whereas a step-by-step comparison might be best for certain exam questions (for example, ‘Discuss the differences between prokaryotes and eukaryotes’). There is often little choice about structure for practical and project reports (see p. 82).

Writing

Adopting a scientific style

Your main aim in developing a scientific style should be to get your message across directly and unambiguously. Although you can try to achieve this through a set of ‘rules’ (see Box 10.1), you may find other requirements driving your writing in a contradictory direction. For instance, the need to be accurate and thorough may result in text littered with technical terms, and the flow may be continually interrupted by references to the literature. The need to be succinct also affects style and readability, for example, through the use of stacked noun-adjectives (such as, ‘restriction fragment length polymorphism’) and acronyms (such as, ‘RFLP’). Finally, style is very much a matter of taste and each tutor, examiner, supervisor or editor will have ‘pet’ loves and hates that you may have to accommodate. Different assignments will need different styles; Box 10.2 gives further details.

Developing technique

Writing is a skill that can be improved, but not instantly. You should analyse your deficiencies with the help of feedback from your tutors. Be prepared to change work habits (for example, start planning your work more carefully; find out more about punctuation), and be willing to learn from some of the excellent texts that are available on scientific writing (p. 74).

Getting started

A common problem is ‘writer’s block’ – inactivity or stalling brought on by a variety of causes. If blocked, ask yourself these questions:

- **Are you comfortable with your surroundings?** Make sure you are seated comfortably at a reasonably clear desk and have minimised the possibility of interruptions and distractions.
- **Are you trying to write too soon?** Have you clarified your thoughts on the subject? Have you done enough preliminary reading?
- **Are you happy with the underlying structure of your work?** If you have not made an outline, try this. If you are unhappy because you cannot think of a particular detail at the planning stage, just start writing – it is more likely to come to you while you are thinking of something else.
- **Are you trying to be too clever?** Your first sentence does not have to be earth-shattering in content or particularly smart in style. A short statement of fact or a definition is fine. If there will be time for

Box 10.1 How to achieve a clear, readable style

- **Words and phrases**

- Choose short, clear words and phrases rather than long ones: e.g. use 'build' rather than 'fabricate'; 'now' rather than 'at the present time'. At certain times, technical terms must be used for precision, but do not use jargon if you do not have to.
- Do not worry too much about repeating words, especially when to introduce an alternative might subtly alter your meaning.
- Where appropriate, use the first person to describe your actions ('We decided to'; 'I conclude that'), but not if this is specifically discouraged by your supervisor/tutor.
- Favour active forms of writing ('the observer completed the survey in ten minutes') rather than a passive style ('the survey was completed by the observer in ten minutes').
- Use tenses consistently. Past tense is always used for Materials and Methods ('samples were taken from. . .') and for reviewing past work ('Smith (1990) concluded that. . .'). The present tense is used when describing data ('Fig. 1 shows. . .'), for generalisations ('Most authorities agree that. . .') and conclusions ('To conclude, . . .').
- Use statements in parentheses sparingly – they disrupt the reader's attention to your central theme (see above).
- Avoid clichés and colloquialisms – they are usually inappropriate in a scientific context.

- **Punctuation**

- Try to use a variety of types of punctuation, to make the text more interesting to read. However, make sure that each punctuation mark assists in getting your meaning across.
- Decide whether you wish to use 'closed' punctuation (frequent commas at the end of clauses) or 'open' punctuation (less frequent commas) – be consistent.
- Do not link two sentences with a comma. Use a full stop, this is an example of what *not* to do.
- Pay special attention to apostrophes, using the following rules:
 - To indicate possession, use an apostrophe before an 's' for a singular word (e.g. the rat's temperature was. . .) and after the s for a plural word ending in s (e.g. the rats' temperatures were = the temperatures of the rats were). If the word has a special plural (e.g. woman → women) then use the apostrophe before the s (the women's temperatures were. . .).

- When contracting words, use an apostrophe (e.g. do not = don't; it is = it's), but remember that contractions are generally *not* used in formal scientific writing.
- Do *not* use an apostrophe for 'its' as the possessive form of 'it' (e.g. the university and its surroundings) Note that 'it's' is reserved for 'it is'. This is an exception to the general rule and a very common mistake.
- Never use an apostrophe to indicate plurals of any kind, including abbreviations.

- **Sentences**

- Do not make them overlong or complicated.
- Introduce variety in structure and length.
- If unhappy with the structure of a sentence, try chopping it into two or more sentences.

- **Paragraphs**

- Get the paragraph length right – five sentences or so. Do *not* submit an essay that consists of a single paragraph, nor one that contains many single sentence paragraphs.
- Make sure each paragraph is logical, dealing with a single subject or theme.
- Take care with the first sentence in a paragraph (the 'topic' sentence); this introduces the theme of the paragraph. Further sentences should then develop this theme, e.g. by providing supporting information, examples, or contrasting cases.
- Use 'linking' words or phrases to maintain the flow of the text within a paragraph (e.g. 'for example'; 'in contrast'; 'however'; 'on the other hand').
- Make your text more readable by adopting modern layout style. The first paragraph in any section of text is usually *not* indented, but following paragraphs may be (by the equivalent of three character spaces). In addition, the space between paragraphs should be slightly larger than the space between lines. Follow departmental guidelines if these specify a format.
- Group paragraphs in sections under appropriate headings and subheadings to reinforce the structure underlying your writing.
- Think carefully about the first and last paragraphs in any piece of writing: these are often the most important as they respectively set the aims and report the conclusions.

Note: If you are not sure what is meant by any of the terms used here, consult a guide on writing (see p. 74).

Box 10.2 How to use appropriate writing styles for different purposes (with examples)

Note that courses tend to move from coursework assignments that are predominantly descriptive in the early years to a more analytical approach towards the final year (see Chapters 3 and 9). Also, different styles may be required in different sections of a write-up, e.g. descriptive for introductory historical aspects, becoming more analytical in later sections.

• Descriptive writing

This is the most straightforward style, providing factual information on a particular subject, and is most appropriate:

- in essays where you are asked to 'describe' or 'explain' (p. 77)
- when describing the results of a practical exercise, e.g.: 'The experiment shown in Figure 1 confirmed that enzyme activity was strongly influenced by temperature, as the rate observed at 37 °C was more than double that seen at 20 °C.'

However, in literature reviews and essays where you are asked to 'discuss' (p. 77) a particular topic, the descriptive approach is mostly inappropriate, as in the following example, where a large amount of specific information from a single scientific paper has been used without any attempt to highlight the most important points:

In a study carried out between July and October 2005, a total of 225 sputum samples from patients attending 25 different clinics in England and Wales were screened. Bacteria were isolated from 67.6% of these samples, with 47.42% of the samples giving *Pseudomonas aeruginosa*, 11.76% *Burkholderia cepacia* and 8.59% *Stenotrophomonas maltophilia* (Grey and Gray, 2009).

In the most extreme examples, whole paragraphs or pages of essays may be based on descriptive factual detail from a single source, often with a single citation at the end of the material, as above. Such essays often score low marks in essays where evidence of deeper thinking is required (Chapter 3).

• Comparative writing

This technique is an important component of academic writing, and it will be important to develop your comparative writing skills as you progress through your course. Its applications include:

- answering essay questions and assignments of the 'compare and contrast' type (p. 77)
- comparing your results with previously published work in the Discussion section of a practical report.

To use this style, first decide on those aspects you wish to compare and then consider the material (e.g. different literature sources) from these aspects – in what ways do

they agree or disagree with each other? One approach is to compare/contrast a different aspect in each paragraph. At a practical level, you can use 'linking' words and phrases to help orientate your reader as you move between aspects where there is agreement and disagreement. These include, for agreement: 'in both cases'; 'in agreement with'; 'is also shown by the study of'; 'similarly'; 'in the same way'; and for disagreement: 'however'; 'although'; 'in contrast to'; 'on the other hand'; 'which differs from'. The comparative style is fairly straightforward, once you have decided on the aspects to be compared. The following brief example compares two different studies using this style:

While Grey and Gray (2019) reported that *Pseudomonas aeruginosa* was present in 47.4% of 225 UK sputum samples, Black and White (2020) showed that 89.1% of sputum samples from 2592 patients were positive for this bacterium.

Comparative text typically makes use of two or more references per paragraph.

• Analytical writing

Typically, this is the most appropriate form of writing for:

- a review of scientific literature on a particular topic
- an essay where you are asked to 'discuss' different aspects of a particular topic
- evaluating a number of different published sources within the Discussion section of a final-year project dissertation.

By considering the significance of the information provided in the various sources you have read, you will be able to take a more critical approach. Your writing should evaluate the importance of the material in the context of your topic (see also Chapter 11). In analytical writing, you need to demonstrate critical thinking (p. 45) and personal input about the topic in a well-structured text that provides clear messages, presented in a logical order and demonstrating synthesis from a number of sources by appropriate use of citations (p. 37). Detailed information and relevant examples are used only to explain or develop a particular aspect, and not simply as 'padding' to bulk up the essay. The following example shows how detail can be used for explanation.

Pseudomonas aeruginosa is often isolated from sputum samples of cystic fibrosis patients: a short-term UK study with a relatively small size sample (225 patients) isolated this bacterium from around half of all samples (Grey and Gray, 2019), while a longer-term study with a far larger sample size (2592 patients) gave an isolation rate of almost 90% (Black and White, 2020).

Analytical writing is based on a broad range of sources, typically with several citations per paragraph.

Box 10.3 How to improve your writing ability by consulting online and written reference works

- **Using dictionaries**

We all know that a dictionary helps with spelling and definitions, but how many of us use one effectively? You should:

- use an online or hardcopy dictionary when in any doubt about spelling or definitions
- prepare a list of words that you have difficulty in spelling; apart from speeding up the checking process, the act of writing out the words helps commit them to memory
- write out a personal glossary of terms. This can help you memorise definitions. From time to time, test yourself.

Not all dictionaries are the same; ask your tutor or supervisor whether he/she has a preference and why. If you feel a hard-copy dictionary would help you, try out the *Oxford Advanced Learner's Dictionary*, which is useful because it gives examples of use of all words and helps with grammar, e.g. by indicating which prepositions to use with verbs. Dictionaries of biology tend to be variable in quality, possibly because the subject is so wide and new terms are continually being coined. *Henderson's Dictionary of Biological Terms* (Benjamin Cummings) is a useful example.

- **Using a thesaurus**

A thesaurus contains lists of words of similar meaning grouped thematically; words of opposite meaning always

appear nearby. The 'synonyms' feature of word processors is the modern-day equivalent. Use these resources to:

- find a more precise and appropriate word to fit your meaning, but check definitions of unfamiliar words
- find a word or phrase 'on the tip of your tongue' by looking up a word of similar meaning
- increase your vocabulary.

There are many websites providing synonyms and antonyms, some of which may be linked to your word processor. *Roget's Thesaurus* is the hard-copy standard. Several publishers produce a combined dictionary and thesaurus.

- **Using guides for written English**

Online and hardcopy guides provide help with the use of words.

- Use them to solve grammatical problems such as when to use 'shall' or 'will', 'which' or 'that', 'effect' or 'affect', 'can' or 'may', etc.
- Use them for help with the paragraph concept and the correct use of punctuation.
- Use them to learn how to structure writing for different tasks.

revision, first get your ideas down on paper and then revise grammar, content and order later.

- **Do you really need to start writing at the beginning?** Try writing the opening remarks after a more straightforward part. For example, with reports of practical work, the Materials and Methods section may be the easiest place to start.
- **Are you too tired to work?** Do not try to 'sweat it out' by writing for long periods at a stretch: stop frequently for a rest.

Reviewing your text

Detailed review of each draft is strongly advised for all writing, apart from in exams, when time for this will be limited. Where possible, schedule your writing so you can leave each draft to 'settle' for at least a couple of days. When you return to it fresh, you will see more easily where improvements can be made. Try the following structured review process, each stage being covered in a separate scan of your text:

1. **Examine content.** Have you included everything you need to? Is all the material relevant?
2. **Check the grammar and spelling.** Can you spot any obvious mistakes?
3. **Focus on clarity.** Is the text clear and unambiguous? Does each sentence really say what you want it to say?

Learning from others – ask a colleague to read through your draft and comment on its content and overall structure.

Reviewing your text – to improve clarity and shorten your text, ‘distil’ each sentence by taking away unnecessary words and ‘condense’ words or phrases by choosing a shorter alternative.

4. **Be succinct.** What could be missed out without spoiling the essence of your work? It might help to imagine an editor has set you the target of reducing the text by 15%.
5. **Improve style.** Could the text read better? Consider the sentence and paragraph structure and the way your text develops to its conclusion.

Common errors

These include (with examples):

- **problems over singular and plural words** (‘a bacteria is’; ‘the results shows’)
- **verbose text** (‘One definition that can be employed in this situation is given in the following sentence’)
- **misconstructed sentences** (‘Health and safety regulations should be made aware of’)
- **misuse of punctuation**, especially commas and apostrophes (for examples, see Box 10.1)
- **poorly constructed paragraphs** (for advice/examples, see Box 10.1).

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STUDY EXERCISES

10.1 'Brainstorm' an essay title. Pair up with a partner in your class. Together, pick a suitable essay title from a past exam paper. Using the spider diagram or another technique, individually 'brainstorm' the title. Meet afterwards, compare your ideas, and discuss their relative merits and disadvantages.

10.2 Improve your writing technique. From the following checklist, identify the *three* weakest aspects of your writing, either in your own opinion or from essay/assignment feedback:

- grammar
- paragraph organisation
- presentation of work
- punctuation
- scientific style
- sentence structure/variety
- spelling
- structure and flow
- vocabulary.

Now either borrow a book from a local library or buy a book that deals with your weakest aspects of writing. Read the relevant chapters or sections and for each aspect write down some tips that should help you in future.

10.3 Improve your spelling and vocabulary with two lists. Create a pair of lists and pin these up beside your desk. One should be entitled *Spelling Mistakes* and the other *New Words*. Now, whenever you make a mistake in spelling or have to look up how to spell a word in a dictionary, add the problem word to your spelling list, showing where you made the mistake. Also, whenever you come across a word whose meaning is unclear to you, look it up in a dictionary and write the word and its meaning in the 'new words' vocabulary list. From time to time, review these lists to see whether your word knowledge has improved.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

11 Writing essays

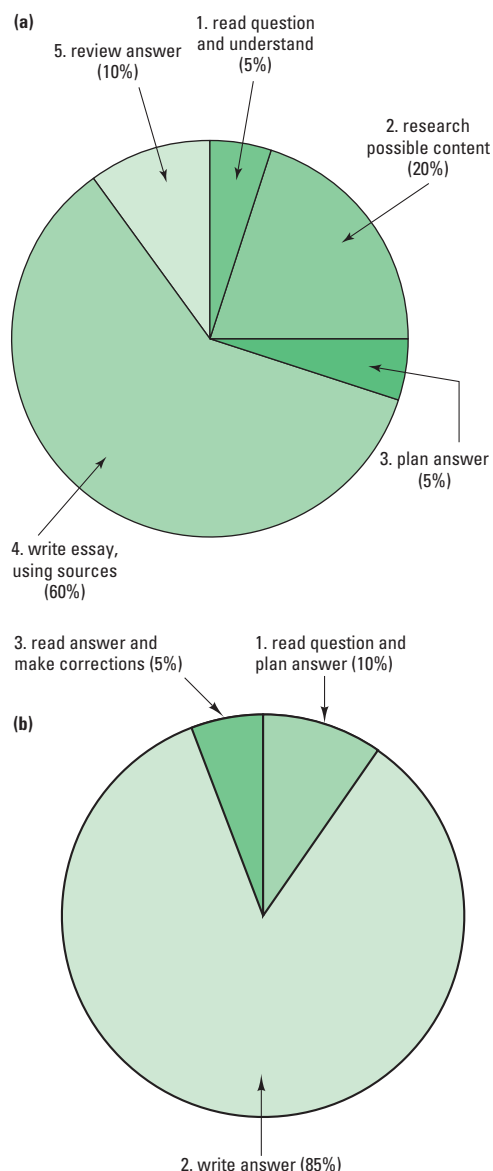


Fig. 11.1 Typical division of time for an essay written (a) as part of in-course assessment or (b) under exam conditions.

The main functions of an essay are to show how much you understand about a topic and how well you can organise and express your knowledge. Assessment then provides a grade or mark that measures your performance, while formative feedback (p. 64) provides you with comments to enable you to improve in subsequent essays.

Organising your time

Your approach will depend on whether you are writing it for in-course assessment or under exam conditions (Fig. 11.1). Essays written over an extended period with access to resources will probably involve a research element, firstly when researching possible content and, secondly, when developing the text and supporting the content with citations (Chapter 5; Fig 11.1(a)). For exams, if you have revised appropriately (Chapter 17), you will have all the necessary information at your fingertips. To keep things uncomplicated, the time allocated for each exam essay should be divided into three components: (i) planning (ii) writing and (iii) reviewing (Fig. 11.1(b)). You should adopt time-saving techniques whenever possible (Box 18.2).

Making a plan for your essay

Begin by dissecting the meaning of the question or title

Read the title very carefully and think about the topic before starting to write. Consider the definitions of each of the important nouns (this can help in approaching the introductory section). Also think about the meaning of verb(s) used and try to follow each instruction precisely (see Table 11.1). Do not get side tracked because you know something about one word or phrase in the title: consider the *whole* title and all of its ramifications. If there are two or more parts to the question, make sure you give adequate attention to each part.

Consider possible content and examples

Revision planning and practice exam essay questions are covered in Chapters 6 and 7. For coursework essays, research content using the methods described in Chapters 4 and 5. Read a range of sources and consider their content in relation to the essay title. Can you spot different approaches to the same subject? Which do you prefer as a means of treating the topic in relation to your title? Which examples are most relevant to your case, and why?

KEY POINT Most marks for essays are lost because the written material is badly organised or is irrelevant. An essay plan, by definition, creates order and, if thought about carefully, should ensure relevance.

Considering essay content – it is rarely enough simply to lay down facts for the reader – you must analyse them and comment on their significance (see p. 17).

Improving your essay writing

These Golden Rules are framed for in-course assessments (p. 64), though many are also relevant to exams (see also Box 18.2).

1. **Read the question carefully**, and decide exactly what the assessor wants you to achieve in your answer.
2. **Make sure you understand the question by considering all aspects** – discuss your approach with colleagues or a tutor.
3. **Carry out the necessary research** (using books, journals, the Internet), taking appropriate notes. Gain an overview of the topic before getting involved in detail.
4. **Always plan your work in outline** before you start writing. Check that your plan covers the main points and that it flows logically.
5. **Introduce your essay** by showing that you understand the topic and stating how you intend to approach it.
6. **Ensure the main content is relevant**, by repeatedly looking back at the question as you write.
7. **Use headings and subheadings** to organise and structure your essay.
8. **Support your statements** with relevant examples, diagrams and references where appropriate.
9. **Conclude by summarising the key points of the topic**, indicating the present state of knowledge, what we still need to find out and how this might be achieved.
10. **Always reread your essay before submitting it**. Check grammar and spelling and confirm that you have answered all aspects of the question.

Table 11.1 Instructions often used in essay questions and their meanings. When more than one instruction is given (e.g. compare and contrast; describe and explain), ensure you carry out both or you may lose marks (see also Table 5.1)

Account for:	give the reasons for
Analyse:	examine in depth and describe the main characteristics of
Assess:	weigh up the elements of and arrive at a conclusion about
Comment:	give an opinion on and provide evidence for your views
Compare:	bring out the similarities between
Contrast:	bring out dissimilarities between
Criticise:	judge the worth of (give both positive and negative aspects)
Define:	explain the exact meaning of
Describe:	use words and diagrams to illustrate
Discuss:	provide evidence or opinions about, arriving at a balanced conclusion
Enumerate:	list in outline form
Evaluate:	weigh up or appraise; find a numerical value for
Explain:	make the meaning of something clear
Illustrate:	use diagrams or examples to make clear
Interpret:	express in simple terms, providing a judgement
Justify:	show that an idea or statement is correct
List:	provide an itemised series of statements about
Outline:	describe the essential parts only, stressing the classification
Prove:	establish the truth of
Relate:	show the connection between
Review:	examine critically, perhaps concentrating on the stages in the development of an idea or method
State:	express clearly
Summarise:	without illustrations, provide a brief account of
Trace:	describe a sequence of events from a defined point of origin

Construct a plan

Every essay needs a structure related to its title. Always write out your plan – but score it through later if written in an exam book. Plan your essay's content in three parts:

1. **The introduction**, which may include definitions and should provide some background information and context for the topic being considered. You should also explain how you plan to approach the subject.
2. **The middle of the essay**, where you develop your answer and provide relevant examples. Decide whether a broad analytical approach is appropriate or whether the essay should contain more factual detail.
3. **The conclusion**, which you can make quite short. You should use this part to summarise and draw together the components of the essay, without merely repeating previous phrases. You might mention such things as: the broader significance of the topic; its future; its relevance to other important areas of biology. Always try to mention both sides of any debate you have touched on, but beware of 'sitting on the fence'.

KEY POINT Use paragraphs to make the essay's structure obvious. Unless otherwise instructed, use headings and subheadings, unless the material beneath the headings would be too short or trivial.

Writing your essay

During this phase, you need to:

- **Maintain your focus on relevance.** Repeatedly ask yourself: 'Am I really answering this question?' Never waffle just to increase the length of an essay. Quality, rather than quantity, is important.
- **Illustrate your answer appropriately.** Use examples to make your points clear, but remember that too many similar examples can stifle the flow of an essay. Use diagrams where a written description would be difficult or take too long. Use tables to condense information (Chapter 73).
- **Take care with presentation.** All essays need to be checked carefully for grammatical and spelling errors. Handwritten exam essays need to be legible – try to cultivate an open form of handwriting, making the individual letters large and distinct.

Using diagrams – give a title and legend and number to each diagram so that it is self-contained and makes sense. Always ensure that you explain in the text when the reader should consult the diagram (e.g. 'as shown in Fig. 1...' or 'as can be seen in the accompanying diagram (Fig. 1),...').

Learning from feedback from lecturers and tutors – ask for further explanation if you do not understand a comment or why an essay was less successful than you thought it should have been.

Reviewing your essay

Make sure that you leave enough time to:

- **Read through the question** to check that you have answered all points;
- **Read through your essay** to check for errors in punctuation, spelling and content. Make any corrections obvious. In an exam do not panic if you suddenly realise you have missed a large chunk out as the reader can be redirected to a supplementary paragraph if necessary.

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STUDY EXERCISES

11.1 Practise dissecting essay titles. Using past exam papers, take each essay title and carefully 'dissect' the wording, working out exactly what you think the assessor expects you to do (see, for example, Table 11.1).

11.2 Write essay plans under self-imposed time limits. Outline plans for essays from a past exam paper. Allow yourself a maximum of 5 minutes per outline. Within this time your main goal is to create an essay plan. To do this, you may need to 'brainstorm' the topic. Alternatively, if you allocate 10 minutes per essay, you may be able to provide more details; for example, list the examples you could describe.

11.3 Practise reviewing your work carefully. For the next assignment you write, review it fully as part of the writing process. This will require you to finish the first draft about one week before the hand-in date; for example, by setting yourself an earlier deadline than the submission date. This exercise is best done with a word-processed essay. Do not worry if it is a little over the word limit at this stage.

- (a) Print out a copy of the essay. Do not look at it for at least two days after finishing this version.
- (b) Review 1: spelling, grammar and sense. Read through the draft critically (try to imagine it had been written by someone else) and correct any obvious errors that strike you. Does the text make sense? Do sentences/paragraphs flow smoothly?
- (c) Review 2: structure and relevance. Consider again the structure of the essay, asking yourself whether you have really answered the question that was set. Are all the parts in the right order? Is anything missed out? Have you followed precisely the instruction(s) in the title? Are the different parts of the essay linked together well?
- (d) Review 3: shorten and improve style. Check the word count. Shorten the essay if required. Look critically at phrasing and, even if the essay is within the word limit, ask yourself whether any of the words are unnecessary or whether the text could be made more concise, more precise or more apt.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

12 Reporting practical and project work

In the early stages of your study, a model format for reporting practical work may be provided, perhaps in the form of a schedule, handout or online document. In later years of study, you may be expected to construct reports yourself. While practical reports, project reports and theses differ greatly in depth, scope and size, they all have the same basic structure, following that adopted in most scientific papers. This usually follows the 'IMRaD' acronym: Introduction, Materials and Methods, Results and Discussion.

KEY POINT Department, school or faculty regulations may specify a particular format for your report or thesis. Consult these rules at an early stage and follow them closely, to avoid losing marks.

Completing practical schedules

A lab schedule will usually give instructions for activities together with spaces for you to add your own observations. Typical activities might include:

- making notes on your lab work
- drawing diagrams of specimens and experimental set-ups
- recording data and results of experiments
- carrying out calculations based on your findings
- carrying out statistical analysis
- outlining your conclusions.

You may be asked to submit your completed schedule for assessment. To produce the best possible work, read through the practical schedule before you attend the practical (if available) and if you anticipate any problems, consult relevant chapters of this book. See, for example, coverage on:

- note-taking (Chapter 4)
- creating tables and graphs (Chapters 73 and 74)
- solving numerical problems (Chapter 75)
- drawing appropriate diagrams (Box 12.1)
- statistical methods (Chapters 76 and 77)
- types of thinking underpinning the conclusions you will be expected to make (Chapters 3 and 6).

Always follow the exact instructions you have been given, both in writing and verbally. Listen carefully to the tips provided by demonstrators and practical leaders, as these may relate to particular specimens used on the day. Where teamwork is expected, perhaps with a lab partner, review the material in Chapter 7 so you can make the most of this partnership.

Preparing practical reports

Being asked to submit a more formal report on your practical work is an exercise designed to make you think more deeply about your lab observations and experiments and to develop the skills necessary for writing up research work. Key features of these practical reports are:

- **Introductory material is generally expected to be short.** Unless otherwise specified, this should simply outline the aims of the experiment(s) with a minimum of background material.
- **Materials and Methods may be provided by your tutor.** If you made changes to this on the day, you should state clearly what you did differently.
- **The accuracy and precision of your data values are important.** These may indicate the care you take with laboratory techniques (for example, weighing or dispensing chemicals, measuring dimensions) and whether you have been able to carry out any necessary calculations without error. Check to see whether your results are of the right order of magnitude and you have provided the correct units.
- **Good presentation of your results is vital.** Diagrams should be accurate and labelled Fig. 12.1, (Box 12.1). Tables and figures need to meet scientific standards (Chapters 73 and 74) and if produced by hand, should be neatly drawn. These aspects are often assessed.
- **A high proportion of marks will be awarded for your analysis of the data.** Make sure your discussion and conclusions show that you understand the rationale behind the experiment, procedure or survey and can be justified by the evidence you present.

Following your tutor's instructions for the content of practical reports – marks will generally be awarded for all of the outcomes and component parts that are mentioned.

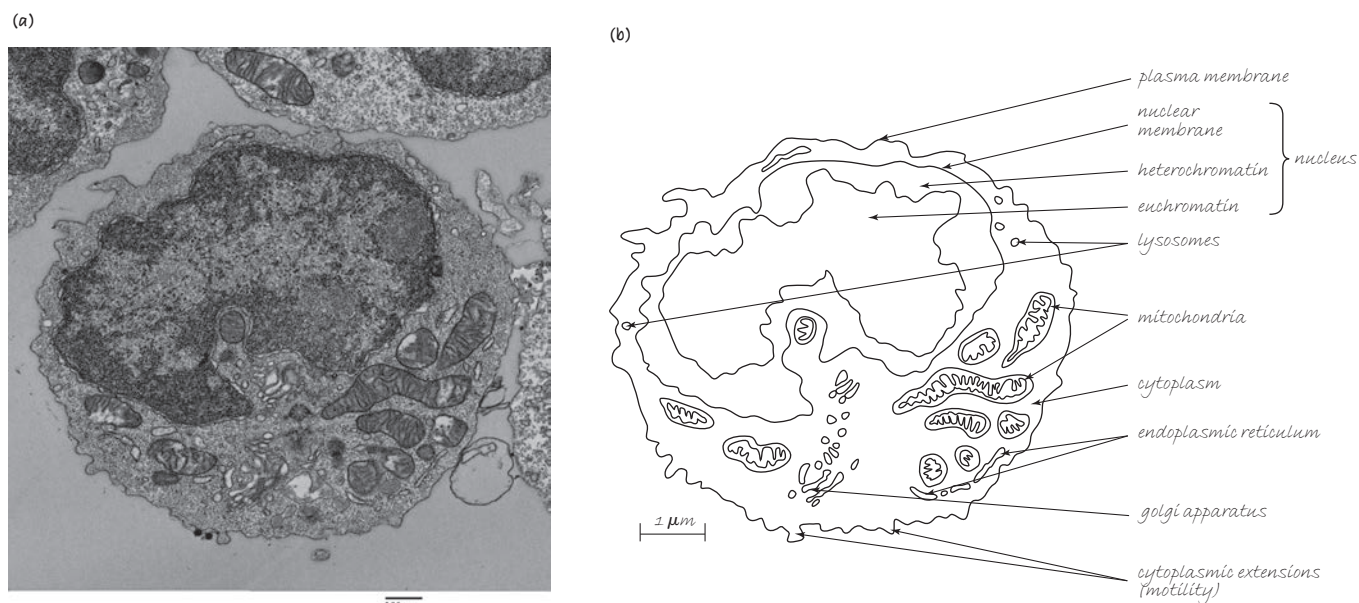


Fig. 12.1 Example of a clearly labelled figure. The labelled cell diagram (Box 12.1) shown in (b) is based on the transmission electron micrograph of a human B lymphocyte (a type of white blood cell) shown in (a). Note that the interpretation of certain structures (e.g. the mitochondria in this example) relies to some extent on background knowledge. Figure 12.1 (a) is used courtesy of the National Institutes of Allergy and Infectious Diseases, National Institutes of Health, under the Creative Commons Attribution 2.0 Generic license, available: <https://creativecommons.org/licenses/by/2.0/deed.en>

Box 12.1 How to create effective diagrams for practical schedules and reports

To produce a high-quality diagram, follow these steps:

1. Decide exactly what you are going to draw and why.

Each type of diagram may require a different approach (see below).

2. Choose how large your diagram should be. In a practical schedule this may depend on the space allotted, and you should aim to use the space fully. In a report, you may have the option of scanning your diagram, importing this into a word-processed file and manipulating the final size.

3. Select where you are going to place your diagram. It can be useful to draw faint 'construction lines' first to ensure that the object(s) drawn are to scale and proportionally correct. Make sure you allow sufficient space for labels.

4. Start drawing. Draw what you see, not what you expect to see. In general, use pencil for most diagrams drawn in practical classes, to allow corrections to be made.

5. Label your diagram carefully and fully. If the diagram is assessed, then accurate (and complete) labelling will be awarded a high proportion of marks. Ask staff if you are unsure about any features you observe.

6. Give your diagram a title, scale and legend. Where appropriate, include organism, classification, part drawn, orientation, stain(s), magnification or scale bar.

Consider the following aspects in relation to the different types of diagram expected:

- **Morphological diagrams and body plans** – drawn by eye, or at low magnification, your main objective is to provide a realistic representation of the specimen, showing the relationships between organs and other components.
- **Tissue diagrams (maps)** – these are usually based on observations from microscope slides. Here, your main purpose is to show how the tissues are organised in a

section through a whole organism or one of its organs. The main difficulty is deciding where to draw the boundary line between tissues: cell differentiation is rarely discrete, so cells at the boundary may show characteristics of both tissues – use your background knowledge to decide positioning.

- **Cell diagrams** – Here, your aim is to show the features of one or more representative cells, allowing you to highlight anatomical differences between specialised types, and correlate these with cell or tissue function. When using prepared slides, it is unlikely that you will be able to identify many subcellular features with confidence, resulting in relatively undetailed diagrams, which may focus on, for example, cell shapes or interrelationships. Using an electron micrograph as a source, you should be able to identify more subcellular features, due both to the extra magnification offered and to specialised staining (Chapter 25). Figures 12.1 (a) and (b) provide examples of a cell diagram based on a TEM image.
- **Apparatus diagrams** – here, your aim is to illustrate the components of an experimental setup. These figures are normally drawn as a 2D section rather than a 3D perspective diagram (see e.g. Fig. 24.2), and you may be more concerned with the relationship between parts than with showing them to a uniform scale.
- **Charts, graphs and histograms** – The main purpose here is to organise and convey information. Charts are useful for illustrating life cycles, metabolic pathways and organisational hierarchies; flowcharts are a specialised form showing the sequence of a complex process (see e.g. Fig. 25.3). To be effective they should be simple, clear and logical in sequence. Make a rough hand-drawn sketch to help you decide on your final layout. Graphs and histograms are used to display numerical information (data) in a way that shows relationships in a visual form. Chapter 74 covers the main types of graphs and explains how they should be constructed.

Preparing project reports

These are submitted as part of the examination for a degree to summarise the outcomes of a lengthy research project (Chapter 33). The report acts to place on record full details of your experimental work and will normally be read by those with a direct interest in it – mainly your supervisor(s) and examiners. Note the following:

- **The Introduction will generally be lengthy.** This should provide the full context for your research, quoting up-to-date references and explaining why the research was done.
- **The Materials and Methods will be detailed.** There needs to be enough information to allow another competent person to be able to repeat your work. Your lab notebook (see p. 126) should provide the basis for writing this section.
- **You are allowed scope to expand on your findings** and to include detail that might be omitted in a scientific paper (Box 12.2).
- **You may have problems organising the amount of information to be reported.** If this is the case, be selective and choose only the important experiments and results; condense data sets using descriptive statistics; and use charts rather than tables to summarise data. Another method of coping with this issue is to divide your thesis into sections or chapters, each having the standard format (as in Box 12.2). A General Introduction can be given at the start and a General Discussion at the end. Discuss this with your supervisor.

Producing a thesis

Choose the observations or experiments you wish to report

Choosing between graphs and tables – graphs are generally easier for the reader to assimilate, whereas tables can be used to condense a lot of data into a small space.

Repeating your experiments – remember, if you do an experiment twice, you have repeated it only once.

Try to start this process before your lab work ends, because at the stage of reviewing your experiments, a gap may become apparent (for example, a missing control) and you might still have time to rectify the deficiency. Irrelevant material should be ruthlessly eliminated, at the same time bearing in mind that negative results can be extremely important (see p. 204). Use as many different forms of data presentation as are appropriate, but avoid presenting the same data in more than one form. Summarise the important points in the text using appropriate descriptive and hypothesis-testing statistics (Chapters 76 and 77), relegating large tables of primary data to an appendix or where available, a digital repository. Make sure that the experiments you describe are representative: always state the number of times they were repeated and how consistent your findings were.

Create plans or outlines for the component parts

The overall structure of practical and project reports is well defined (see Box 12.2), but individual parts will need to be well organised, as with any other form of scientific writing (see Chapter 10). The main optional variants of the general structure include combining Results and Discussion into a single section and adding a separate Conclusions section.

The main advantage of a joint Results and Discussion section is that you can link together different experiments, perhaps explaining why a particular result led to a new hypothesis and the next experiment. However, a combined Results and Discussion section may contravene your department's regulations, so you should check before using this approach. The main advantage of having a separate Conclusions section is to draw together and emphasise the chief points arising from your work, when these may have been 'buried' in an extensive Discussion section.

Box 12.2 How to structure reports of experimental work

Undergraduate project reports are generally modelled on this arrangement or a close variant of it, because this is the structure used for nearly all research papers and theses. In scientific papers, a list of Keywords (for computer cross-referencing systems) may be included following the Abstract. Acknowledgements may appear after the Contents section, rather than near the end. Department or faculty regulations for producing theses and reports often require a Title Page to be inserted at the start and a List of Figures and Tables as part of the Contents section, and may specify declarations and statements to be made by the student and supervisor.

Part (in order)	Contents/purpose	Checklist for reviewing content
Title	Explains what the project was about	<input type="checkbox"/> Does it explain what the text is about succinctly? <input type="checkbox"/> Are all the details correct?
Authors plus their institutions	Explains who did the work and where; also where they can be contacted now	
Abstract/Summary	Synopsis of methods, results and conclusion of work described.	<input type="checkbox"/> Does it explain why the work was done? <input type="checkbox"/> Does it outline the whole of your work and your findings?
Contents	Shows the organisation of the text (not required for short papers)	<input type="checkbox"/> Are all the sections covered? <input type="checkbox"/> Are the page numbers correct?
Abbreviations	Lists all the abbreviations used (but not those of SI, chemical elements, or standard biochemical terms)	<input type="checkbox"/> Have they all been explained? <input type="checkbox"/> Are they all in the accepted form? <input type="checkbox"/> Are they in alphabetical order?
Introduction	Orientates the reader, explains why the work has been done and its context in the literature, why the methods used were chosen, why the experimental organisms were chosen. Indicates the central hypothesis behind the experiments	<input type="checkbox"/> Does it provide enough background information and cite all the relevant references? <input type="checkbox"/> Is it of the correct depth for the readership? <input type="checkbox"/> Have all the technical terms been defined? <input type="checkbox"/> Have you explained why you investigated the problem? <input type="checkbox"/> Have you outlined your aims and objectives? <input type="checkbox"/> Have you explained your hypothesis and methodological approach?
Materials and Methods	Explains how the work was done. Should contain sufficient detail to allow another competent worker to repeat the work	<input type="checkbox"/> Is each experiment covered, avoiding unnecessary duplication? <input type="checkbox"/> Is there sufficient detail to allow repetition of the work? <input type="checkbox"/> Are proper scientific names and authorities given for all organisms? <input type="checkbox"/> Have you explained where you got the organisms from? <input type="checkbox"/> Are the correct names, sources and grades given for all chemicals?
Results	Displays and describes the data obtained. Should be presented in a form that is easily assimilated (graphs rather than tables, small tables rather than large ones)	<input type="checkbox"/> Is the sequence of experiments logical and adequately linked? <input type="checkbox"/> Are the data presented in the clearest possible way? <input type="checkbox"/> Have SI units been used properly throughout? <input type="checkbox"/> Has adequate statistical analysis been carried out? <input type="checkbox"/> Is all the material relevant? <input type="checkbox"/> Are figures and tables all numbered in order? <input type="checkbox"/> Are their titles appropriate? <input type="checkbox"/> Do the figure and table legends provide all the information necessary to interpret the data without reference to the text? <input type="checkbox"/> Have you presented the same data more than once?
Discussion/Conclusions	Discusses the results: their meaning, their importance; compares the results with those of others; suggests what to do next	<input type="checkbox"/> Have you explained the significance of the results? <input type="checkbox"/> Have you compared your data with other published work? <input type="checkbox"/> Are your conclusions justified by the data presented?
Acknowledgements	Gives credit to those who helped carry out the work	<input type="checkbox"/> Have you listed everyone that helped, including any grant-awarding bodies?
Literature cited (Bibliography)	Lists all references cited in appropriate format: provides enough information to allow the reader to find the reference in a library	<input type="checkbox"/> Do all the references in the text appear on the list? <input type="checkbox"/> Do all the listed references appear in the text? <input type="checkbox"/> Do the years of publications and authors match? <input type="checkbox"/> Are the journal details complete and in the correct format? <input type="checkbox"/> Is the list in alphabetical order, or correct numerical order?

Following the expected format –

additional parts may be specified for dissertations and theses: a Title Page is often required and a List of Figures and Tables as part of the Contents section. In some cases, you may need to include a declaration about the authorship and originality of the work.

Presenting your results – remember that the order of results presented in a report need not correspond with the order in which you carried out the experiments: you are expected to rearrange them to provide a logical sequence of findings.

Using the correct tense – always use the past tense to describe the methodology used in your work, since it is now complete. Use the present tense only for generalisations and conclusions (p. 71).

Definition

Peer review – the process of evaluation of scientific work, where a paper is reviewed by two or more expert reviewers ('referees'), with comments on quality and significance as a key component of the validation procedure.

Write

The Materials and Methods section is often the easiest to write once you have decided what to report. Remember to use the past tense and do not include results or discussion. The Results section is the next easiest as it should only involve description. At this stage, you may benefit from jotting down ideas for the Discussion – this may be the hardest part to compose as you need an overview both of your own work and of the relevant literature. It is also liable to become wordy, so try hard to make it succinct. The Introduction should not be too difficult if you have fully understood the aims of the experiments. Write the Abstract and complete the list of references at the end. To assist with the latter, it is a good idea to collate appropriately formatted details of the references (Chapter 5) as you progress.

Revise the text

Once your first draft is complete, try to answer all the questions given in Box 12.2. Show your work to your supervisors and learn from their comments. Let a friend or classmate who is unfamiliar with your subject read your text; they may be able to pinpoint obscure wording and show where information or explanation is missing. If writing a thesis, double-check that you are adhering to your institution's thesis regulations.

Prepare and submit the final version

Markers appreciate neatly produced work but a well-presented document will not disguise poor science. Make sure figures are clear and in the correct size and format. Produce the final version with the best print quality option available.

Your department will specify when to submit a thesis, so plan your work carefully to meet this deadline or you may lose marks. Tell your supervisor early of any circumstances that may cause delay and check to see whether any forms must be completed for late submission, or evidence of extenuating circumstances.

Producing a scientific paper

Scientific papers are the means by which research findings are communicated to others. Peer-reviewed papers are published in journals, many of which are online; each covers a well-defined subject area and provides details of the format they expect.

KEY POINT Peer review is an important component of the process of scientific publication; only those papers whose worth is confirmed by the peer-review process will be published.

It would be very unusual for an undergraduate science student to submit a paper on their own – this would normally be done in collaboration with a project supervisor and only then if your research has satisfied appropriate criteria. However, it is important to know how research papers come into being (Box 12.3), as this can help you understand and interpret the primary literature (Chapter 5).

Box 12.3 How to produce a scientific paper

Scientific papers are the lifeblood of science and it is useful to understand how these are produced. The main steps include the following:

1. **Assessing potential content** – The work must be of an appropriate standard to be published and should be 'new, true and meaningful'. Therefore, before starting, the authors need to review their work critically under these headings. The material included in a scientific paper will generally be a subset of the total work done during a project, so it must be carefully selected for relevance to a clear central hypothesis – if the authors will not edit, the referees and editors of the journal will return the work for revision and editing.
2. **Choosing a journal** – The main factors in deciding on an appropriate journal are the range of subjects it covers, the quality of its content and the number and geographical distribution of its readers. The choice of journal always dictates the format of a paper since authors must follow to the letter the journal's 'Instructions to Authors'. This applies to both printed and online journals.
3. **Deciding on authorship** – In multi-author papers, a contentious issue is often who should appear as an author and in what order they should be cited. Where authors make an equal contribution, an alphabetical order of names may be used. Otherwise, each author should have made a substantial contribution to the paper and should be prepared to defend it in public. Ideally, the order of appearance will reflect the amount of work done rather than seniority. This may not always happen.
4. **Writing** – The paper's format will be similar to that shown in Box 12.2, and the process of writing will include outlining, reviewing, etc., as discussed elsewhere in this chapter. Figures must be produced to an appropriate standard and any images must be of very high quality.
5. **Submitting** – When completed, copies of the paper are submitted to the editor of the chosen journal with a simple covering letter. A delay of one to two months usually follows while the manuscript is sent to two or more anonymous referees who will be asked by the editor to check that the paper is novel, scientifically correct and that its length is justified.
6. **Responding to referees' comments** – The editor will send on the referees' comments to the authors who will then have a chance to respond. The editor will decide on the basis of the comments and replies to them whether the paper should be published or not, or whether it should be revised and resubmitted.
7. **Checking proofs and waiting for publication** – If a paper is accepted, it will be sent off to the publishers. The next the authors see of it is the proofs (first printed version in style of journal), which have to be checked carefully for errors and returned. Eventually, the paper will be published. A delay of several months following acceptance is not unusual for printed papers, but most papers are now available electronically, via the Web, in a shorter time.

Sources for further study

Davis, M. and Davis, K.J. (2012) *Scientific Papers and Presentations. Navigating Scientific Communication in Today's World*, 3rd edn. Academic Press.

Gastel, B. and Day, R.A. (2017) *How to Write and Publish a Scientific Paper*, 8th edn. Cambridge University Press, Cambridge.

Hofmann, A.H. (2019) *Scientific Writing and Communication: Papers, Proposals, and Presentations*, 4th edn. Oxford University Press, New York

Lobban, C.S. and Schefter, M. (2005) *Successful Lab Reports: A Manual for Science Students*. Cambridge University Press, Cambridge.

Luey, B. (2011) *Handbook for Academic Authors*, 5th edn. Cambridge University Press, Cambridge.

Matthews, J.R., Bowen, J.M. and Matthews, R.W. (2015) *Successful Science Writing: A Step-by-step Guide for the Biological and Medical Sciences*, 4th edn. Cambridge University Press, Cambridge.

McMillan, K.M. and Weyers, J.D.B. (2014) *How to Complete a Successful Research Project*. Pearson Education, London.

Valiela, I. (2009) *Doing Science: Design, Analysis and Communication of Scientific Research*, 2nd edn. Oxford University Press, Oxford.

[Covers scientific communication, graphical presentations and aspects of statistics.]

Wang, G.T. and Park, K. (2016) *Student Research and Report Writing: From Topic Selection to the Complete Paper*, Wiley-Blackwell, Hoboken.

STUDY EXERCISES

12.1 Write a formal 'Materials and Methods' section.

Adopting the style of a research paper (e.g. past tense, all relevant detail reported such that a competent colleague could repeat your work), write out the Materials and Methods for a practical you have recently carried out. Ask a colleague or tutor to comment on what you have written.

12.2 Describe a set of results in words.

Again adopting the style of a research paper, write a paragraph describing the results contained in a particular table or graph. Ask a

fellow student or tutor to comment on your description, to identify what is missing or unclear.

12.3 Write an abstract for a paper.

Pair up with a fellow student. Each of you should independently choose a different research paper in a current journal. Copy the paper, but mask over the abstract section, having first counted the words used. Swap papers. Now, working to the same amount of words as in the original, read the paper and provide an abstract of its contents. Then, compare this with the real abstract.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

13 Tackling literature surveys and reviews

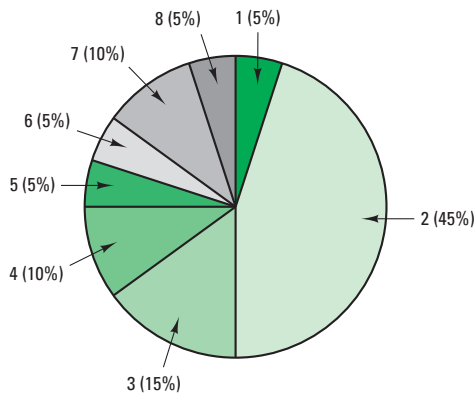


Fig. 13.1 Pie chart showing how you might allocate time for a literature survey:

1. select a topic
2. scan the literature
3. plan the review
4. write first draft
5. leave to settle
6. prepare a structured review of text
7. write final draft
8. produce top copy.

Creating a glossary – one barrier to developing an understanding of a new topic is the language used – jargon and specialised terminology. To overcome this, create your own glossary. You may wish to cross-reference a range of sources to ensure your definitions are reliable and context-specific. Remember to note your sources in case you wish to use the definition within your review.

Using index cards – these can help you organise information for large numbers of references. Write key points and citation information on each card – this helps when considering where the reference fits into the literature. Arrange the cards in themed piles, eliminating irrelevant ones. Order the piles in the sequence in which you wish to write, then use each pile to help you organise your thoughts for writing.

A literature survey or review (sometimes described as a ‘dissertation’) is a specialised form of essay that summarises and reviews the evidence and concepts for a particular area of research. They are used as assignments to test your ability to use higher cognitive and writing skills (Chapters 3 and 10), to analyse published results and synthesise new ideas about a topic.

KEY POINT A literature review is more than a simple recitation of facts. The best reviews analyse and evaluate information rather than simply describe it.

Organising your time

Figure 13.1 illustrates how you might divide up your time for writing a literature survey. There are many subdivisions in this chart because of the size of the task: in general, for lengthy tasks, it is best to divide up the work into manageable chunks. Note also that proportionately less time is allocated to writing itself than with an essay. In a literature survey, make sure that you spend adequate time on research and revision.

Selecting a topic

You may have no choice in the subject area to be covered, but if you do, carry out your selection of a topic as a three-stage process:

1. **Identify a broad subject area that interests you.**
2. **Find and read relevant literature in that area.** Try to gain a broad impression of the field from books and general review articles. Decide which areas particularly interest you. Discuss your ideas with your supervisor/tutor.
3. **Select a relevant and concise title.** The wording should be considered very carefully as it will define the content expected by the reader. A narrow subject area will cut down on the amount of literature you will be expected to review, but will also restrict the scope of the conclusions you can make – and vice versa for a wide subject area.

Scanning the literature and assessing your sources

You will need to carry out a thorough investigation of the literature *before* you start to write. The key issues are as follows:

- **Get an initial toe-hold in the literature.** Seek help from your supervisor, who may be willing to supply details or copies of a few key papers to get you started. Hints on expanding your collection of references are given on p. 35.
- **Assess the relevance and significance of each article.** This is the essence of writing a review, but it is difficult unless you already have a good understanding of the field. Try reading earlier reviews in your area and discussing the topic with your supervisor/tutor or other academic staff.
- **Clarifying your thoughts.** One approach is to use the SPSEER method (Box 13.1). Another way to gain a better overview of a complex topic is to subdivide the literature into smaller subject areas.

Box 13.1 How to analyse a topic using the SPSER approach

This method is useful when trying to get to grips with a new and complex subject. The approach helps you to 'deconstruct' or 'unpack' the topic and involves considering the task in five discrete stages given the acronym SPSER. Your thoughts could be noted using a table or the 'Pattern' or 'Mind Map' approaches (p. 26), ready for converting into text.

1. **Situation.** Briefly outline the context of the topic and the history of its development. For some complex topics you may also need to refer to an introductory text or Website to build a foundation for your understanding.
2. **Problem.** State the essential issue(s) or problem(s) that researchers or others are tackling. In a research paper, this might be stated succinctly in the Abstract, but you

might also find it at the end of the Introduction. In some cases this will be obvious from the title of the exercise you have been given.

3. **Solution.** Outline possible solutions or ways of tackling the problem. These will probably arise from your reading of papers, reviews and other sources.
4. **Evaluation.** Note positive or negative aspects of each solution and provide evidence to support your viewpoint. A tabular approach might be useful, listing positives on one side and negatives on the other.
5. **Recommendation.** Arrive at a conclusion by deciding what might/should happen next, outlining your reasoning.

Defining your terms – the Introduction is a good place to explain the meaning of the key terms used in your survey or review.

Balancing opposing views – even if you favour one side of a disagreement in the literature, your review should provide a balanced and fair description of *all* the published views of the topic. Having done this, if you do wish to state a preference, give reasons for your opinion.

Deciding on structure and content

The general structure of a literature survey is described below. The *Annual Review* series (available in most university libraries) provides good examples of the style expected in formal reviews of the biosciences.

Introduction

The Introduction should give the general background to the research area, concentrating on its development and importance. You should also make a statement about the scope of your survey; as well as defining the subject matter to be discussed, you may wish to restrict the period being considered.

Main body of text

The review should discuss the published work in the selected field and may be subdivided into appropriate sections. Within each section, the approach is usually chronological, with appropriate linking phrases (for example, 'Following on from this, . . .'; 'Meanwhile, Smith (2020) tackled the problem from a different angle . . .'). However, a good review is much more than a chronological list of work done. It should:

- **Allow the reader to obtain an overall view of the current state of the research area**, identifying the key areas where knowledge is advancing.
- **Show how techniques are developing** and discuss the benefits and disadvantages of using particular organisms, methods or experimental systems.
- **Assess the relative worth of different types of evidence** – this is the most important aspect (see Chapter 6). Do not hold back from taking a critical approach as the conclusions you may read in the primary literature are not always correct.
- **Indicate where there is conflict in findings or theories**, suggesting, if possible, which is the stronger case.
- **Indicate gaps in current knowledge** – include possible approaches to resolving these gaps, where appropriate.

You do not need to wait until you have read all the sources available to you before starting to write the main body. Word processors allow you to

modify and move pieces of text at any point and it will be useful to write paragraphs about key sources, or groups of related papers, as you read them (Chapter 14). Nevertheless, you should try to create a general plan for your review as soon as possible. Place your draft sections of text under an appropriate set of subheadings that reflects your plan, but be prepared to rearrange these and retitle or reorder sections as you proceed. Not only will working in this way help to clarify your thoughts, but it may help you avoid a last-minute rush of writing near to the submission date.

Conclusions

Your conclusions should draw together the threads of the preceding parts and point the way forward, perhaps listing areas of ignorance or where the application of new techniques may lead to advances. This section is one of the most important parts of the review, and should not be neglected or rushed.

References and other necessary information

The References or Literature Cited section should provide full details of all papers referred to in the text (see p. 38). The regulations for your department may also specify the format and position for other material, such as a title page, contents page and list of acknowledgements.

Making citations – a literature review poses stylistic problems because of the need to cite large numbers of papers; in the *Annual Review* series this is overcome by using numbered references, rather than using the author (year) format for citations. However, if a format is specified, you should always follow this.

Sources for further study

McMillan, K.M. and Weyers, J.D.B. (2011) *How to Write Dissertations and Project Reports*, 2nd edn. Prentice Hall, Harlow.

Rudner, L.M. and Schafer, W.D. (1999) How to write a scholarly research report, *Practical Assessment*,

Research & Evaluation, 6(13). Available: <https://files.eric.ed.gov/fulltext/ED435712.pdf>
Last accessed 27/04/21.
[See also the sources for further study noted at the end of Chapter 12.].

STUDY EXERCISES

13.1 Summarise the main differences between a review and a scientific paper. From the many subject areas in the *Annual Review* series (find via your library's periodical indexing system), pick one that matches your subject interests, and within this find a review that seems relevant or interesting. Read the review and write down *five* ways in which the writing style and content differs from that seen in scientific papers.

13.2 Gather a collection of primary sources for a topic. From the journal section of the library, select an interesting scientific paper published about 5–10 years ago. First, work *back* from the references cited by that paper: can you identify from the text or the article

titles which are the most important and relevant to the topic? List five of these, using the proper conventions for citing articles in a reference list (see Chapter 5). Note that each of these papers will also cite other articles, always going back in time. Now, using a citation index (see p. 36), work *forward* and find out who has cited your selected article in the time since its publication. Again list the five most important articles found.

13.3 Write a synopsis of a review. Using one of the *Annual Review* series as a source, allow yourself just *five* single-sentence bullet points to summarise the key points reported in a particular review.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

14 Presenting written assignments

Reassessing your IT abilities in relation to your course – try to gain a picture of what the expectations of staff are by referring to the learning objectives/outcomes, then assess your current expertise in relevant IT areas. You will probably be familiar with the fundamentals of 'office' style software (e.g. Microsoft Office or Apple Pages), but even when you know the basics of a software program, learning about advanced features can ensure you are working more efficiently and effectively.

How you produce and present your work influences the grades you achieve, because a proportion of the total marks will probably be allocated to this aspect. A wide range of skills is involved in writing and submitting your work, such as organisation, phrasing, layout and printing. Whatever the methods used, you should ensure that your assignments follow the conventions and specifications for different types of coursework (Chapters 11–13).

Making the most of word processing features

These programs offer specific advantages for academic work, allowing you to:

- **Adapt your methods to longer writing exercises** – you can, for example, set up an outline structure at the beginning and create new text in any sequence as you go along. You can refine material many times before submission and insert new material easily. You can also create a bibliography at the outset and add new references as you consult them (Chapters 5 and 10).
- **Use editing features to refine your writing** – you can cut and paste blocks of text to create a better flow for your material. You can make your writing more accurate and easier to read and avoid errors by using features such as spelling and grammar checkers and the inbuilt thesaurus. If working to a specific word limit, you can also use the word count feature to check your progress and ensure you do not under- or over-run.
- **Present documents and their components in particular formats** – you should use these to follow the relevant instructions for your assignment, including fonts, text size, line spacing, margins, paragraph layout, table layout and figure styles. Although you can reformat your text at any time, it is good practice to enter the relevant details in the 'Page setup' when you start writing: entering them later can lead to layout problems.
- **Produce a high-quality submission** – you can check the design and layout of your work before printing or submitting it electronically.


Box 14.1 covers further aspects of the creation and presentation of documents.

KEY POINT It is vital to save your work frequently to a memory stick, hard drive or network drive. This should be done every 15 minutes or so, or you may lose hours of work. As part of this process, you may wish to develop a systematic file naming system for the ongoing versions.

Take full advantage of the differences between word processing and handwriting. This might include:

- **Inserting your initial ideas for a plan**, for example, at the level of paragraph topics. The order can be altered easily and if the paragraph grows too long, it can easily be split.

Box 14.1 How to use the features of word processors to produce your assignment

- **Customisation** – most word processors provide several routes to achieve the same result. These include: standard menus and commands, customisable toolbar features and key combinations. Examples of useful customised commands relevant to scientific writing include subscripts and superscripts, word count and A–Z sort.
- **Spellchecker and thesaurus** – these usually suggest US spellings as default – adjust as appropriate. Standard spellcheckers may indicate a misspelling for technical terms. You may wish to check the word's spelling and add it to the dictionary file. Do not rely on the spellchecker to spot all errors: they cannot pick up words that are incorrect but are legitimate words e.g. 'their' for 'there', or 'form' for 'from'. Where alternative words are found with a thesaurus, perhaps to avoid repetition, make sure that the meaning is truly equivalent to the word you are replacing.
- **Grammar checker** – this feature highlights text that may be incorrectly structured. In general, the guidance is unreliable for scientific writing except when pointing out obvious errors, such as a missing verb. Academic style frequently demands the use of impersonal language involving passive tense and this is usually marked up as an error, when it is perfectly acceptable in this context.
- **Cut and paste** – moving blocks of text is very valuable when reviewing and editing, and has changed the nature of writing as you can write sections out of sequence, according to availability of information or personal interest. However, there is a risk that the planning phase of writing (Chapters 10–13) will be neglected, resulting in weak structure. To counteract this, create a plan and use temporary or permanent headings to organise your writing.
- **Find and replace** – the 'find' (or 'search') procedure allows you to scan quickly through a document looking for a specified word, phrase or punctuation. This is valuable when combined with a replace facility so that, for example, you could replace the word 'test' with 'trial' throughout your document simply and rapidly. The key combination **CTRL** + F is a useful alternative to the menu command for 'find'.
- **Format painter and repeat** – these functions allow you to copy the format of one section to another. The 'redo' or 'repeat' command is equally valuable where repeated formatting or text entry is required.
- **Lists** – keep to simple bullet designs and be consistent with your choice of indentation.
- **Footnotes** – if required, these can be added. A sequential numeric or alphabetic superscript is generated next to the chosen word and the footnote is then entered either at the foot of the page or at the end of the document.
- **Headers, footers and page numbering** – these allow you to identify your document and in large documents the relevant section or chapter (as in this textbook). Page numbers can be inserted automatically, generally as part of the footer. It is normal not to number the first page.
- **Fonts and document layout** – these aspects are often defined in the instructions for submission but if not, you should consider the following:
 - **Typefaces:** these fall into three broad groups: serif fonts with curves and flourishes at the ends of the characters (e.g. Times New Roman, the most commonly used for standard text); sans-serif fonts without such flourishes, providing a clean, modern appearance (e.g. Arial, as used in this Box); and decorative fonts used for special purposes only, such as the production of newsletters and notices. Try not to mix typefaces too much in a formal document.
 - **Font size:** measured in units called points. There are about 28 points per cm. The standard sizes for text are 10, 11 and 12 point, but typefaces are available up to 72 point or more e.g. for headings.
 - **Font colour:** in general, only black fonts, lines and symbols are used in academic documents, including tables and graphs. However, colour may be used for text in poster displays and slides for spoken presentations (Chapters 15 and 16).
 - **Margins:** a typical A4 layout for a text document might require a 2.5 cm left margin and about 2 cm top, bottom, and right.
 - **Headings:** if allowed, these are useful to divide your text into topics and they may also be specified (e.g. as part of reports, such as Introduction, Materials and Methods – see Chapter 12). You can add contrast to headings by using a bold sans-serif typeface. The font size should not differ greatly for different levels of headings, and should be compatible with the text font size.
 - **Line spacing and paragraph indentation:** use double or 1.5 line spacing for printed assignments, to allow space for the marker to add written comments or corrections. You may wish to add extra blank lines before headings and additional half lines between paragraphs to improve readability. Paragraph indentation may be specified – a common format is not to indent the first paragraph in a section, but to indent thereafter (as here), but increasingly 'blocked' paragraphs are used with no indents. Create indentations using the **TAB** or  key rather than the space bar.
 - **Justification:** this is the term describing the way in which text is aligned vertically. Left justification is normal, but for formal documents, both left and right justification may be used.

Avoiding 'widow' and 'orphan' lines – these are single lines at the top and bottom of a page respectively and are generally undesirable in page design. You can control whether they appear in most word processors by specifying page breaks or using the widow/orphan control feature.

Using specialised presentation packages for graphics – programs that can enhance your presentation include:

- **SigmaPlot**, which can produce graphs with floating axes
- **Adobe Illustrator** which is useful for designing complex graphics
- **Adobe Dreamweaver** which enables you to produce high-quality Web pages
- **MindGenius** which can be used to produce Mind Maps.

Important points regarding the use of such packages are:

- **the learning time required for some of the more complex operations can be considerable**
- **default or 'chart wizard' settings for graphs may result in output that is unacceptable for the sciences**
- **default fonts in labels and legends may be inconsistent with other parts of your presentation**
- **there may be difficulties in producing some symbols**, e.g. Greek letters such as μ (do not use 'u' as a substitute). The same applies to scientific notation and superscripts (do not use 14C for ^{14}C , and replace, e.g., 1.4E + 09 with 1.4×10^9) – try cutting and pasting symbols from Word.

Avoiding citation and copyright issues – take care to address these matters if using the images of others (see Chapter 5).

- **Starting to write by laying down basic ideas and concepts**, filling in the details and refining the language later.
- **Focussing first on content rather than grammar, spelling and word repetition.** These aspects can (and should) be checked during a separate, subsequent revision of your text.
- **Carrying out multiple editing phases** to improve the content and language.
- **Setting screen view to 'print layout'** so you can review the text in its final layout.
- **Using voice-controlled writing.** Programs such as Siri and Cortana allow you to 'dictate' text into a word processor compatible file. This can save time and effort, but only after you have 'trained' the software – you will also need to proofread carefully, and correct any transcription errors.

Constructing tables and graphics

Tables

These are widely used in the biosciences, but the conventions for their presentation are specific (see Box 73.1). So, while you can produce basic tables by accessing 'quick tables' formats, this is rarely appropriate for formal submissions. Your main options here are to:

- **Use the inbuilt table-constructing commands.** Here, the basic table outline can be adjusted to fit your needs: for example, by changing numbers of columns and rows, altering column widths, merging cells or deleting boundary lines.
- **Use a spreadsheet or database (Chapter 72) to construct the table and then copy it to the word processor file.** This requires considerably more manipulation than using the word processor directly. It is best reserved for special circumstances, such as very large or complex tables of data, and especially where calculations or recalculations are involved and/or the data are already stored as a spreadsheet.

Special characters and equations

You can draw lines and insert other small-scale graphical features such as brackets directly from the keyboard and special characters (for example, mathematical symbols and Greek characters) are available from the *Symbols* menu. An *Equation Editor* may be available on your setup to assist with mathematical formulae – consult the help menu for guidance. When laying out equations, these are usually indented on a separate line and, where appropriate, numbered in square brackets. Examples are shown in many chapters of this book (see, for example, p. 144).

Diagrams, graphs and images

Word processors can be used to generate simple charts and diagrams. More complex diagrams can be produced in presentation software such as *PowerPoint*, where it is easier to create the elements to suit your needs and then move them around. For scientific graphs, it is better to construct these with a fully featured spreadsheet, such as *Excel* (see Box 74.2) and then import the final graph into the text of your document.

Fig. 14.1 Layout and content of a typical cover sheet for an assignment.

KEY POINT The 'default' graphs generated by 'office' programs are rarely satisfactory for scientific presentation. You will need to make appropriate changes to meet scientific standards and style. Box 74.1 gives a checklist for graph drawing and Box 74.2 provides guidelines for adapting *Excel* output.

Printing and submitting your document

Always leave a reasonable amount of time to review your writing and presentation (Chapter 10). The appearance of a document on-screen generally represents what the printout will look like, but this should be checked using the 'print preview' feature before printing large documents, or where your paper size is non-standard. If you prefer, use a draft printout for review. This can sometimes reveal errors not easily seen on screen, although it will not include any grammar checking 'flags' from the word processor program. Most printers offer choices as to text and graphics quality, so choose draft or 'eco' quality for all but your final copy. Remember to include a cover sheet as the first page of your submission (Fig. 14.1).

Often, the production of the final version of your assignment will be close to the assessment deadline. Avoid the temptation to keep refining your document until the last possible moment. Late assignments generally incur a scaled penalty, so try to avoid a last-minute rush through good planning.

KEY POINT Make sure you know well beforehand *where and how* your document is to be submitted. This could be an office location for printed copies or digital dropbox for online submission. Allow time for delivery or the relevant file transfer processes.

Sources for further study

Anon. *Wikiversity: Computer Skills/Basic/Word Processing*. Available: https://en.wikiversity.org/wiki/Computer_Skills/Basic/Word_Processing
Last accessed 27/04/21.
[Source of basic multimedia tutorials and information, with links to more advanced tutorials.]

Anon. *Writing university assignments*. Available: <https://www.open.edu/openlearn/ocw/mod/oucontent/view.php?id=19203&printable=1>
Last accessed: 27/04/21

[This is part of an Open University course, which also provides other useful guidance.]

Last accessed 27/04/21.

Gecawich, M. (2017) *27 Word Processing Skills All Students Should Know*. Available: <https://blog.iacademy.com/computer-apps/27-word-processing-skills-students-know/>
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Neville, C. (2008) *How to Improve your Assignment Results*. Open University Press, Maidenhead.

STUDY EXERCISES

14.1 Investigate intermediate/advanced Word features.

The tasks in the following list are likely to be useful in preparing assignments and report writing within the life sciences. Can you carry out all of the tasks? If not, use either a manual or the online *Help* feature to find out how to accomplish them. Tips are given in the answer section.

- Sort information in a list into alphabetical order.
- Replace a text string word or phrase with a new text string throughout your document.
- Replace a text string in normal font with the same text string in italics throughout your document.
- Add a 'header' and 'footer' to your document, the former showing the document's title and the latter containing page numbers on the bottom centre of the page.
- Adjust the margins of the page to give a 5 cm margin on the left and a 2 cm margin on the right.
- Change the type of bullets used in a list from standard (● or ■) to a different form (e.g. -, ◆ or ☑).
- Use the 'thesaurus' option to find a different or more suitable word to express your meaning. Try, for example, to find alternatives to the word 'alternative'.

- Carry out a spellcheck on your document.
- Carry out a word count on your document and on a selected part of it.
- Open two documents and switch between them.

14.2 Make precise copies of tables. Copy the following tables using a word processor such as *Word*.

Test organism	Results of analysis (units)		
	August	September	October
X			
Y			
Z			

Results of analysis (units)			
Test organism	August	September	October
X			
Y			
Z			

14.3 Investigate what programs and packages are available to you as a student. Test each program with appropriate data, images, etc.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

15 Preparing a poster

Learning from others – look at the various types of posters around your university and elsewhere; the best examples will be visual, not textual, with a clear structure that helps get the key messages across.

A scientific poster is a visual display of the results of an investigation, usually mounted on a rectangular board. Posters are used to display project results or assignment work in undergraduate courses, and to communicate research findings at scientific meetings.

In a written report you can include a reasonable amount of specific detail and the reader can go back and reread difficult passages. However, if a poster is long-winded or contains too much detail, your reader is likely to lose interest.

KEY POINT Think of a poster session as a competition – you are competing for the attention of people in a room. Because you need to attract and hold the interest of your audience, make your poster as interesting as possible. Think of it as an advertisement for your work and you will not go far wrong.

Checking the details

Before considering the content of your poster, you should find out:

- **the dimensions of your poster area**, typically up to 1.5 m wide by 1 m high
- **the composition of the poster board** and the method of attachment, whether pins, Velcro[®] tape, or some other form of adhesive; and whether these will be provided – in any case, it is safer to bring your own
- **the time(s) when the poster should be set up and when you should attend**
- **the room where the poster session will be held.**

Designing your poster

Plan your poster with your audience in mind, as this will dictate the appropriate level for your presentation. Aim to make your poster accessible to a broad audience. Since a poster is a *visual* display, you must pay particular attention to the presentation of information: work that may have taken hours to prepare can be ruined in a few minutes by the ill-considered arrangement of items (Fig. 15.1). Begin by making a draft sketch of the major elements of your poster. It is worth discussing your intended design with someone else, as constructive advice at the draft stage will save a lot of time and effort when you prepare the final version (or consult the Sources for further study on p. 100).

Choosing a layout

One approach is to divide the poster into several smaller areas, perhaps six or eight in all, and prepare each as a separate item on a piece of card. Alternatively, you can produce a single large poster on one sheet of paper or card and store it inside a protective cardboard tube. However, a single large poster may bend and crease, making it difficult to flatten out. In addition, photographs and text attached to the backing sheet may work loose; a large poster with embedded images is an alternative approach.

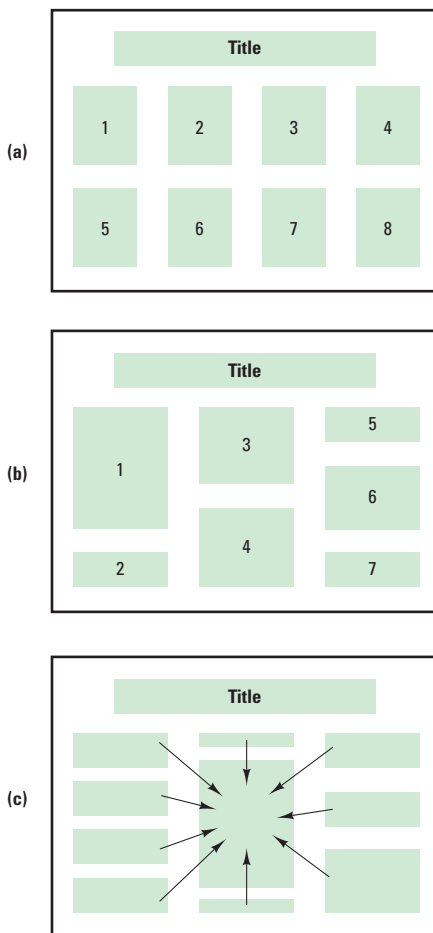


Fig. 15.1 Poster design. (a) An uninspiring design: subunits of equal area, reading left to right, are not recommended. (b) This design is more interesting and the text will be easier to read (column format). (c) Another approach, with a central focus and arrows to guide the reader.

Presenting a poster at a formal conference – it can be useful to include your photograph for identification purposes, e.g. in the top right-hand corner of the poster.

Assembling your poster – text and graphics printed on good-quality paper can be glued directly onto a contrasting mounting card: use photographic spray mountant or double-sided tape, rather than liquid glue. Trim carefully using a guillotine to give equal margins, parallel with the paper. Place photographs in a window mount to prevent their corners from curling. Another approach is to trim pages or photographs to their correct size, then laminate in plastic film: this gives a highly professional, protective finish and is easy to transport.

Subdividing your poster means that each smaller area can be prepared on a separate piece of paper or card, of A4 size or slightly larger, making transport and storage easier. It also breaks the reading matter up into smaller pieces, looking less formidable to a potential reader. By using pieces of card of different colours you can provide emphasis for key aspects, or link text with figures or photographs.

You will need to guide your reader through the poster using headings/subheadings to help with this aspect. It may be appropriate to use either a numbering system, with large, clear numbers at the top of each piece of card, or a system of arrows (or thin tapes), to link sections within the poster (see Fig. 15.1). Make sure that the relationship is clear and that the arrows or tapes do not cross.

Selecting a title

Your title should be concise (no more than eight words), specific and interesting, to encourage people to read the poster. Make the title large and bold – it should run across the top of your poster, in letters at least 4 cm high, so that it can be read from the other side of the room. Coloured spirit-based marker and block capitals drawn with a ruler work well, as long as your writing is readable and neat (the colour can be used to add emphasis). Alternatively, you can print out each word in large type, using a word processor. Details of author(s) should be given, usually across the top of the poster in somewhat smaller lettering than the title.

Writing the text

Write in short sentences and avoid verbosity. Keep your poster as visual as possible and make effective use of the spaces between the blocks of text. Your final text should be double-spaced and should have a minimum capital-letter height of 8 mm (minimum type size 36 point), preferably greater, so that the poster can be read at a distance of 1 m. One method of obtaining text of the required size is to photo-enlarge standard typescript (using a good-quality photocopier), or use a high-quality printer. It is best to avoid continuous use of text in capitals, since it slows reading and makes the text less interesting to the reader. Also avoid italic, informal or decorative styles of lettering.

KEY POINT Keep text to a minimum – aim to have a *maximum* of 500 words in your poster.

Using subtitles and headings

These should have a capital-letter height of 12–20 mm, and should be restricted to two or three words. They can be produced by word processor, photo-enlargement, by stencilling, or by hand, using pencilled guidelines (but make sure that no pencil marks are visible on your finished poster).

Choosing colours

Consider the overall visual effect of your chosen display, including the relationship between your text, diagrams and the backing board. Colour can be used to highlight key aspects of your poster. However, it is very easy to ruin a poster by the inappropriate choice and application of colour. Careful use of two, or at most three, complementary colours and shades

Producing composite material for posters

– *PowerPoint* is generally more useful than *Word* when you wish to include text, graphics and/or images on the same page. It is possible to use *PowerPoint* to produce a complete poster (Box 15.1), although it can be expensive to have this printed out commercially to A1 or A0 size.

Designing the materials and methods section

– photographs or diagrams of apparatus can help to break up the text of this section and provide visual interest. It is sometimes worth preparing this section in a smaller typeface.

Keeping graphs and diagrams simple

– avoid composite graphs with different scales for the same axis, or with several trend lines (use a maximum of three trend lines per graph).

Listing your conclusions – a series of numbered points is a useful approach, if your findings fit this pattern.

will be easier on the eye and should aid comprehension. Colour can be used to link the text with the visual images (for example, by picking out a colour in a photograph and using the same colour on the mounting board for the accompanying text). For *PowerPoint* posters, careful choice of colours for the various elements will enhance the final product (Box 15.1). Use coloured inks or water-based paints to provide colour in hand-drawn diagrams and figures, as felt pens rarely give satisfactory results.

Tackling content

The typical format for a research project poster is that of a scientific report (see Box 12.2), i.e. with the same headings, but with a considerably reduced content. Keep references within the text to a minimum – readers can always ask you for further information. Also note that posters often have a summary/conclusions section at the end, rather than an abstract.

Preparing the introduction

This should give the reader background information on the broad field of study and the aims of your own work. It is important that this section is as interesting as possible, to capture the interest of your audience. It is often worth listing any specific objectives as a series of numbered points.

Describing the materials and methods

Keep this short, and describe only the principal techniques used. You might mention any special techniques, or problems of general interest.

Presenting the results

Do not present your raw data: use data reduction wherever possible, i.e. figures and simple statistical comparisons. Graphs, diagrams, histograms and pie charts give clear visual images of trends and relationships and should be used in place of data tables (see p. 576). Final copies of all figures should be produced so that the numbers can be read from a distance of 1 m. Each should have a concise title and legend, so that it is self-contained: if appropriate, a series of numbered points can be used to link a diagram with the accompanying text. Where different symbols are used, provide a key on each graph (symbol size should be at least 5 mm). Avoid using graphs straight from a written version, for example, a project report, textbook, or a paper, without modification to meet your requirements.

Stating the conclusions

This is where many readers will begin. This needs to be the strongest part of your poster, summarising the main points. Refer to your figures here to draw the reader into the main part of your poster. A slightly larger or bolder typeface may add emphasis, though too many different typefaces can look messy. For references, smaller type can be used in a final section.

Taking part in a poster session

A poster session may be organised as part of the assessment of your coursework or research project, and this usually mirrors those held at most scientific conferences and meetings. Staff and fellow students (equivalent to the delegates at a conference) will mill around, looking at the posters and chatting to their authors, who are usually expected to be in attendance. If you stand at the side of your poster throughout, you are

Providing a handout – this could be a useful way to summarise the main points of your poster, so that your readers have a permanent record of the information you have presented.

likely to discourage some readers, who may not wish to become involved in a detailed conversation about the poster before they have read it through. Stand nearby. Find something to do – talk to someone else, or browse among the other posters, but remain aware of people reading your poster and be ready to answer any queries raised. Do not be too discouraged if you are not asked lots of questions: remember, the poster is meant to be a self-contained, visual story, without need for further explanation.

Box 15.1 How to create a poster using PowerPoint

Software such as *PowerPoint* can be used to produce a high-quality poster, providing you have access to a good colour printer. However, you should avoid the standard templates available on the Web as they encourage unnecessary uniformity and stifle creativity, leading to a less satisfying end-result. The following steps give practical advice on creating a poster as a single *PowerPoint* slide.

- 1. Sketch out your plans.** Decide on the main poster elements (images, graphs, tables and text sections) and their relationship with each other and draw out a one-page 'storyboard' (see Fig. 15.1). Think about colours for background, text and graphics (use two or three complementary colours) – dark text on a light background is clearer (high contrast), and uses less ink when printing. Also consider how you will link the elements in sequence, to guide readers through your 'story'.
- 2. Get your material ready.** Collect together individual files for pictures, figures and tables. Make any required adjustments to images, graphs or tables before you import them into your poster.
- 3. Create a new/blank slide.** Use the software's formatting commands to create a slide with the appropriate orientation (portrait or landscape) and size (e.g. A4, A3). Use the templates or select an appropriate background style and colour. In general, avoid setting a picture as your background as these tend to detract from the content of the poster, unless you 'fade out' the detail (increase opacity). Save your work frequently and in more than one location (e.g. hard drive and USB memory stick).
- 4. Add graphics.** For images, use the software's commands to choose and insert the correct file. Alternatively, use the copy-and-paste functions of complementary software. Once inserted, resize using the 'sizing handles' in one of the corners (for photographs, take care not to alter one dimension relative to the other, or the image will be distorted). While the software offers standard shapes and other useful features, you should avoid clipart (jaded and overused) and poor-quality images from the Internet (always use the highest resolution possible, making sure that you attribute all such images and that you are not infringing copyright) – if you do not have your final images in the early stages, use blank text boxes to show their position within the poster.
- 5. Add your text.** Select a *Text Box* and place this on your slide, then either type in or copy-and-paste your text from a word-processed file. You will need to consider the font size for the printed poster (e.g. for an A0 poster (size 1189 × 841 mm), a printed font size of around 24 point is appropriate for the main text, with larger fonts for headings and titles. If you find things difficult to read on-screen, use the *Zoom* function. Use a separate text box for each element of your poster and do not be tempted to type too much text into each box – write in phrases, using bullet points and numbered lists to keep text concise (aim for no more than 50 words per text box). Select appropriate font styles and colours. Use software formatting commands for background colour or surrounding lines. Present supplementary text elements in a smaller font – for example, details of methodology, references cited.
- 6. Add boxes, lines and/or arrows** to link elements of the poster and guide the reader (e.g. Fig. 15.1). Note that new inserts are overlaid on older inserts – if this proves to be a problem, select the relevant item and move it forwards or backwards.
- 7. Review your poster.** Get feedback from another student or tutor, e.g. on a small printed version, or use a projector to view your poster without printing (adjust the distance between projector and screen to give the correct size).
- 8. Revise and edit your poster.** Revisit your work and remove as much text as possible. Delete any component that is not essential to the message of the poster. Keep graphs simple and clear (p. 573 gives further advice). White space is important in providing structure and distance between elements.
- 9. Print the final version.** Use a high-resolution colour printer (this may be costly, so you should wait until you are sure that no further changes are needed).

Coping with questions in assessed poster sessions – you should expect to be asked questions about your poster, and to explain details of figures, methods, etc. Think about possible questions beforehand, and be prepared to answer these in the session.

A poster display will never feel like an oral presentation (Chapter 16), where the nervousness beforehand is often replaced by a combination of satisfaction and relief as you unwind after the event. However, a poster can be an effective means of communication, particularly if you follow these guidelines.

Sources for further study

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STUDY EXERCISES

15.1 Design a poster. Working with one or more partners from your year group, decide on a suitable topic (perhaps something linked to your current teaching programme). Working individually, make an outline plan of the major elements of the poster, with appropriate subheadings and a brief indication of the content and relative size of each element (including figures, diagrams and images). Exchange draft plans with your partners and arrange a session where you can discuss their merits and disadvantages.

15.2 Prepare a checklist for assessing the quality of a poster presentation. After reading through this chapter, prepare a 10-point checklist of assessment

criteria under the heading 'What makes a good poster presentation?'. Compare your list with the one that we have provided (see Website below) – do you agree with our criteria, or do you prefer your own list (can you justify your preferences)?

15.3 Evaluate the posters in your university. Most universities have a wide range of academic posters on display. Some may cover general topics (e.g. course structures), while others may deal with specific research topics (e.g. poster presentations from past conferences). Consider their good and bad features (if you wish to make this a group exercise, you might compare your evaluation with that of other students in a group discussion session).

Answers to these study exercises are available at go.pearson.com/uk/he/resources

16 Giving a spoken presentation

Practising 'speaking skills' – opportunities include:

- answering lecturers' questions
- contributing in tutorials
- talking to informal groups
- giving your views at formal (committee) meetings
- demonstrating or explaining to other students, e.g. during a practical class
- asking questions in lectures/seminars
- answering an examiner's questions in an oral exam.

Learning from experience and the mistakes of others – use your own experience of good and not-so-good lecturers and other presenters to shape your approach and performance. Some of the more common errors to avoid include:

- speaking too quickly
- reading from notes or from slides and ignoring the audience
- unexpressive, impersonal or indistinct speech
- distracting mannerisms
- poorly structured material with little emphasis on key information
- factual information too complex and detailed
- using too few or too many visual aids.

Testing the room – if possible, try to rehearse your talk in the room in which it will be presented. This will help you to make allowance for layout of equipment, lighting, acoustics and sight lines that might affect the way you deliver your talk. It will also put you more at ease on the day, because of the familiarity of the surroundings.

Most students feel very nervous about giving talks. This is natural, since very few people are sufficiently confident and outgoing that they look forward to speaking in public. Additionally, the technical nature of the subject matter may give you cause for concern, especially if you feel that some members of the audience have a greater knowledge than you have. This is further amplified when tutors and/or peers are assessing your 'performance'. However, delivering a spoken presentation is a fundamental method of scientific communication and an important transferable skill; therefore, it forms an important component of many courses.

The comments in this chapter apply equally to coursework presentations, for example, those based on assignments and project work, and to formal presentations at scientific conferences. It is hoped that the advice and guidance given below will encourage you to make the most of your opportunities for public speaking, but there is no substitute for practice. Do not expect to find all of the answers from this, or any other, book. Rehearse, repeat and learn from your own experience.

KEY POINT The three 'Rs' of successful public speaking are: *reflect* – give sufficient thought to all aspects of your presentation, particularly at the planning stage; *rehearse* – to improve your delivery; *revise* – modify the content and style of your material in response to your own ideas and to the comments of others.

Preparing your presentation

Preliminary information

Begin by establishing the details needed to plan your presentation, including:

- **the duration of the talk**
- **whether time for questions is included/expected**
- **the size and location of the room**
- **the projection/lighting facilities provided**, and whether pointers or similar aids are available.

It is especially important to find out whether the room has the necessary equipment for digital projection (for example, PC/laptop, projector and screen, black-out curtains or blinds, appropriate lighting) or other means of display before you prepare your audio-visual aids. If you concentrate only on the spoken part of your presentation at this stage, you are inviting trouble later on. Have a look around the room and try out the equipment at the earliest opportunity, so that you are able to use the lights, projector, etc., with confidence.

Visual aids

These are used to support your talk with images, using a 'show and tell' format. For digital projection systems, check that you can upload and display your material in the location where you will give your presentation. Box 16.1 gives general advice on using presentation software packages such as Microsoft *PowerPoint*.

Box 16.1 How to prepare and use presentation software such as Microsoft PowerPoint in a spoken presentation

Microsoft PowerPoint and similar programs such as Apple Keynote can be used to produce high-quality visual aids, assuming a computer and digital projector are available in the room where you intend to speak. The presentation is produced as a series of electronic 'slides' on to which you can insert images, diagrams and text. In contrast, software such as Prezi allows you to take a more holistic approach, where you assemble the components of your talk on a single 'canvas' that allows you to pan and zoom to different parts at different points in the presentation. When creating a slide-based presentation slides, bear the following points in mind:

- **Plan the structure of your presentation.** Decide on the main topic areas and sketch out your ideas on paper. Think about what material you will need (e.g. pictures, graphs) and what colours to use for background and text.
- **Choose slide layouts according to purpose.** You can add material to each new slide to suit your requirements.
- **Select your background with care.** Many of the preset background templates of presentation software are best avoided, since they are overused and fussy, diverting attention from the content of the slides. Moreover, flat, dull backgrounds may seem uninteresting, while brightly coloured backgrounds can be garish and distracting. Choose whether to present your text as a light-coloured type on a dark background (more restful but perhaps less engaging if the room is dark) or a dark-coloured type on a light background (more lively).
- **Use visual images throughout.** Remember the maxim 'a picture is worth ten thousand words'. A presentation composed entirely of text-based slides will be uninteresting: adding images and diagrams will brighten up your talk. Images can be taken with a digital camera, scanned in from a printed version or copied and pasted from the Web, but you should take care not to break copyright regulations (p. 38). 'Clip art' is copyright-free, but should be used sparingly, as they are rarely wholly relevant and trivialise your presentation. Diagrams graphs and tables can be imported from other programs, e.g. Excel (Box 16.1 gives further specific practical advice on adding graphics, saving files, etc.).
- **Keep text to a minimum.** Aim for no more than 20 words on a single slide (e.g. four/five lines containing a few words per line). Use headings and subheadings to structure your talk: write only key words or phrases as 'prompts' to remind you to cover a particular point during

your talk – never be tempted to type whole sentences as you will then be reduced to reading these from the screen during your presentation, which is boring.

- **Use a large, clear font.** Set your default font to a non-serif style such as Arial, or Verdana. Default fonts for headings and bullet points are intentionally large, for clarity. Do not reduce these to anything less than 28-point type size (preferably larger), in order to cram in more words: if you have too much material, create a new slide and divide up your information.
- **Animate your material.** Animation enables you to introduce the various elements within a slide in a sequence that fits with your narrative. For example, text can be made to appear one line at a time, to prevent the audience from reading ahead, helping to maintain their attention.
- **Do not overdo the special effects.** Presentation software has a wide range of features that allow complex slide transitions and animations, additional sounds, etc., but these quickly become irritating to an audience unless they have a specific purpose within your presentation.
- **Always edit your slides before use.** Check through your slides and cut out any unnecessary words, adjust the layout and animation. Remember the maxim 'less is more' – avoid too much text; too many bullet points; too many distracting visual effects and sounds.

When presenting your talk:

- **Work out the basic procedures beforehand.** Practise, to make sure that you know how to move forwards and backwards, turn the screen on and off, hide the mouse pointer, etc.
- **Do not forget to engage your audience.** Despite the technical gadgetry, you need to play an active role in their presentation, as explained elsewhere in this chapter. Always remember that slides are there to illustrate a presentation, not to replace the speaker.
- **Do not go too fast.** Sometimes, new users tend to deliver their material too quickly: try to speak at a normal pace, and practise beforehand.
- **Consider whether to provide a handout.** Presentation software often has several options for handouts, including some that provide space for notes (e.g. Fig. 4.1). However, a handout should not be your default option, as it may distract your audience and there are production costs involved.

Using visual aids – do not let equipment and computer gadgetry distract you from the essential rules of good speaking (Box 16.2). Remember that *you* are the presenter and everything else plays a supporting role.

Pitching your talk at the right level – the general rule should be: ‘do not overestimate the background knowledge of your audience’. This sometimes happens in student presentations, where fears about the presence of ‘experts’ can encourage the speaker to include too much detail, overloading the audience with facts.

Getting the introduction right – it can be a good idea to have an initial slide giving your details and the title of your talk, and a second slide telling the audience how your presentation will be structured. Make eye contact with all sections of the audience during the introduction.

Deciding what to cover in your introductory remarks – you should:

- explain the structure of your talk
- set out your aims and objectives (p. 7)
- explain your approach to the topic.

Identifying your audience

You should consider your audience at the earliest stage, since they will determine the appropriate level for your presentation. If you are talking to fellow students you may be able to assume a common level of background knowledge. In contrast, a research lecture given to your department, or a paper at a meeting of a scientific society, will be presented to an audience from a broader range of backgrounds. An oral presentation is not the place for a complex discussion of specialised information: build up your talk from a low level. The speed at which this can be done will vary according to your audience. As long as you are not boring or patronising, you can cover basic information without losing the attention of the more knowledgeable members in your audience.

Considering your content

Although the specific details in your talk are for you to decide, most spoken presentations share some common features of structure, as described below.

Introductory remarks

It is vital to capture the attention of your audience at the outset. Consequently, you must make sure your opening comments are strong, otherwise your audience will lose interest before you reach the main message. Remember it takes a sentence or two for an audience to establish a relationship with a new speaker. Your opening sentence should be some form of preamble and should not contain any key information. For a formal presentation, you might begin with ‘Thank you for that introduction. My talk today is about . . .’ then restate the title and acknowledge other contributors, etc. You might show a transparency or slide with the title printed on it, or an introductory photograph, if appropriate. This should provide the necessary settling-in period for your audience.

After these preliminaries, you should introduce your topic. Begin your story on a strong note – avoid timid or apologetic phrases.

Opening remarks are unlikely to occupy more than 10% of the talk. However, because of their significance, you might reasonably spend up to 25% of your preparation time on them.

KEY POINT Make sure you have practised your opening remarks so that you can deliver the material in a flowing style, with fewer chances of mistakes.

The main message

This section should include the bulk of your experimental results or literature findings, depending on the type of presentation. Keep details of methods to the minimum needed to explain your data. This is *not* the place for a detailed description of equipment and experimental protocol (unless it is a talk about methodology). Results should be presented in an easily digested format.

Allowing time for slides – as a rough guide you should allow at least two minutes per illustration, although some diagrams may need longer, depending on content and complexity. Make a note of the half-way point to help you check timing/pace during the presentation.

KEY POINT Do not expect your audience to cope with large amounts of data; use a maximum of six numbers per slide. Remember that graphs and diagrams are usually better than tables of raw data, since the audience will be able to see the visual trends and relationships in your data.

Present summary statistics (Chapters 76 and 77) rather than individual results. Show the final results of any analyses in terms of the statistics calculated, and their significance (p. 609), rather than dwelling on details of the procedures used. Figures should not be crowded with unnecessary detail. Every diagram should have a concise self-contained title and the symbols and trend lines should be clearly labelled, with an explanatory key where necessary. When presenting graphical data (Chapter 74) always ‘introduce’ each graph by stating the units for each axis and describing the relationship for each trend line or data set.

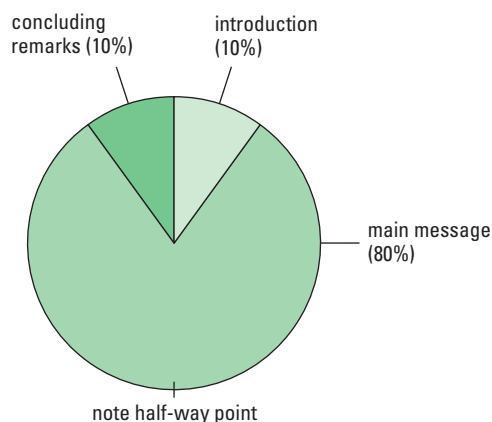


Fig. 16.1 Pie chart showing time allocation for a typical presentation.

KEY POINT Use summary slides at regular intervals, to maintain the flow of the presentation, to emphasise the main points and to ensure that your audience understands the ‘take-home message’.

Take the audience through your story step by step at a reasonable pace. Try not to rush the delivery of your main message due to nervousness. Avoid complex, convoluted storylines – one of the most distracting things you can do is to fumble backwards through slides to try and find something. If you need to use the same diagram or graph more than once then you should make two (or more) copies. In a presentation of experimental results – such as a talk about a final-year project – you should discuss each point as it is raised, in contrast to written text, where the results and discussion may be in separate sections. The main message typically occupies approximately 80% of the time allocated to an oral presentation (Fig. 16.1).

Concluding remarks

Having captured the interest of your audience in the introduction and given them the details of your story in the middle section, you must now bring your talk to a conclusion. Do not end weakly, for example, by running out of steam on your last slide. Provide your audience with a clear ‘take-home message’ by returning to the key points in your presentation. It is often appropriate to prepare a slide listing your main conclusions as a numbered series.

Signal the end of your talk by saying ‘finally . . .’, ‘in conclusion . . .’, or a similar comment and then finish speaking after that sentence. Your audience will lose interest if you extend your closing remarks beyond this point. You may add a simple end phrase (for example, ‘thank you for listening’) as you put your notes away, but do not say anything flippant such as ‘that’s all folks!’, or any similar offhand remark. Finish as strongly and as clearly as you started. Box 16.2 gives further advice.

Considering your final remarks – make sure you give the audience sufficient time to assimilate your final slide: some of them may wish to write down the key points. Alternatively, you might provide a one-page handout, with a brief outline of the aims of your study and the major conclusions.

Box 16.2 How to improve your spoken presentations

In planning the delivery of your talk, bear the following aspects in mind:

- **Using notes.** Many accomplished speakers use abbreviated notes for guidance, rather than reading word-for-word from a prepared script. When writing your talk:
 - Consider preparing your first draft as a full script: write in spoken English and keep the text simple, to avoid a formal, impersonal style. Your aim should be to *talk* to your audience, not to *read* to them.
 - If necessary, use note-cards with key words and phrases: it is best to avoid using a full script in the final presentation. As you rehearse and your confidence improves, a set of note-cards may be an appropriate format. Mark the position of slides/key points, etc.: each note-card should contain details of structure as well as content. Your notes should be written/printed in text large enough to be read easily during the presentation (also check that the lecture room has a lectern light or you may have problems reading your notes if the lights are dimmed). Each note-card or sheet should be clearly numbered, so that you can follow them in sequence, and do not lose your place.
 - Decide on the layout of your talk: give each subdivision a heading in your notes, so that your audience is made aware of the structure.
 - Memorise your introductory/closing remarks: you may prefer to rely on a full written version for these sections, in case your memory fails, or if you suffer 'stage fright'.
 - Using *PowerPoint* and similar presentation software: here, you can either use the 'notes' option, or you may even prefer to dispense with notes entirely, since the slides will help structure your talk, acting as an aide-mémoire for your material.
- **Working on your timing.** It is essential that your talk is the right length and the correct pace:
 - Rehearse your presentation: ask a friend to listen and to comment constructively on those parts that were difficult to follow, to improve your performance.
 - Use 'split times' to pace yourself: following an initial run-through, add the times at which you should arrive at the key points of your talk to your notes. These timing marks will help you keep to time during the final presentation.
 - Avoid looking at your wristwatch when speaking; this sends a negative signal to the audience. Use a wall clock (where available), or take off your watch and put it beside your notes so that you can glance at it without distracting your audience.
- **Considering your appearance.** Make sure that the image you project is appropriate for the occasion:
 - Think about what to wear: aim to be respectable without 'dressing up', otherwise your message may be diminished.
 - Develop a good posture: it will help your voice projection if you stand upright, rather than slouching, or leaning over a lectern.
 - Deliver your material with expression: project your voice towards the audience at the back of the room and make sure you look round to make eye contact with all sections of the audience. Arm movements and subdued body language will help maintain the interest of your audience. However, you should avoid extreme gestures (it may work for some TV personalities but it is not recommended for the beginner).
 - Try to identify and control any repetitive mannerisms: repeated 'empty' words/phrases, fidgeting with pens, keys, etc., will distract your audience. Note-cards held in your hand give you something to focus on, whereas laser pointers will show up any nervous hand tremors. Practising in front of a mirror may help.
- **Thinking about questions the audience may ask.** Once again, the best approach is to prepare beforehand:
 - Consider what questions are likely to come up, and then prepare brief answers. However, do not be afraid to say 'I don't know': your audience will appreciate honesty, rather than vacillation, if you do not have an answer for a particular question.
 - If no questions are asked, you might pose a question yourself and then ask for opinions from the audience: if you use this approach, you should be prepared to comment briefly if your audience has no suggestions, to avoid the presentation ending in an embarrassing silence.

Sources for further study

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STUDY EXERCISES

16.1 Prepare a checklist for assessing the quality of an oral presentation. After reading through this chapter, prepare a 10-point checklist of assessment criteria under the heading 'What makes a good oral presentation?'. Compare your list with the one that we have provided (see website) – do you agree with our criteria, or do you prefer your checklist? Can you justify your preferences?

16.2 Evaluate the presentation styles of other speakers. There are many opportunities to assess the strengths and weaknesses of academic 'public speakers',

including your university lecturers, seminar speakers, presenters of TV documentaries, etc. Decide in advance how you are going to tackle the evaluation (e.g. with a quantitative marking scheme, or a less formal procedure).

16.3 Rehearse a talk and get feedback on your performance. There are a number of approaches you might take, including: (i) recording and reviewing your presentation using a smartphone or digital camera; or (ii) giving your talk to a small group of fellow students and asking them to provide constructive feedback.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

17 Revision strategies

Where your studies are assessed through an exam, you will need to prepare for this by revising and, because you may have a number of exams in a short period, you will need to be well organised. You will need to match your study effort with the expectations of your lecturers, as explained in lectures and course materials, and through the feedback you have been given on previous assignments and exams. Adopting active methods of revision that are suited to your approach to learning (Chapter 3) can also make a significant difference to your performance.

KEY POINT Use the learning objectives/outcomes (p. 33) for your module/course (normally published on the module/course website or in the relevant handbook) as a fundamental part of your revision planning. These explain what you will be expected to be able to do after taking part in the course, so exam questions are often closely based on them. Check this by reference to past papers – see if you can align the questions with the learning objectives.

Preparing for revision – researching the exam format

Before you start revising, find out as much as you can about each exam, including:

- its format and duration
- the date and location
- the types of questions
- whether any questions/sections are compulsory
- whether the questions are internally or externally set or assessed
- whether the exam is ‘open book’, and if so, which texts or notes are allowed.

Self-managing when revising – this is vital to success. When preparing for a revision period, review the tips in Chapter 2 regarding planning, time-management and workspace organisation.

Your course tutor is likely to give you details of exam structure and timing beforehand, so that you can plan your revision; the module handbook and past papers (if available) can provide further useful details (Box 17.1). *Always check that the nature of the exam has not changed before you make plans based on past papers.*

Organising and using lecture notes and marked coursework

Given their importance as a source of material for revision, you should sort out any deficiencies in your lecture notes and practical reports at an early stage. For example, you may have missed a session due to illness, etc., but the exam is likely to assume attendance throughout the year. Ask your classmates for copies of any notes you may have missed.

Your marked practical reports and assessed coursework assignments will contain specific comments from the teaching staff, indicating where you gained marks, alongside details of corrections, errors, and so on. Most lecturers are prepared to discuss such details with students on a one-to-one basis and this information may provide you with insights into the expectations of individual lecturers that may be useful in exams set by the same members of staff. However, you should never ‘fish’ for specific information on possible exam questions, as this will not be well received.

Filing lecture notes – make sure your notes are kept neatly and in sequence by using a ring binder system for hard copies, or a suitable set of folders and filenames for electronic notes.

Box 17.1 How to use past exam papers in your revision

These are a valuable resource; use them in your revision through the following steps:

- 1. Find out where the past (or sample) exam papers are located.** Copies may be lodged online – for example in your university's VLE – or in hard-copy format in the library.
- 2. Locate and copy relevant papers for your module(s).** Check with your tutor or course handbook that the style of paper will not change for the next set of exams.
- 3. Analyse the design of the exam paper.** Taking into account the length in weeks of your module, and the different lecturers and/or topics for those weeks, note any patterns that emerge. For example, can you translate weeks of lectures/practicals into numbers of questions or sections of the paper? Consider how this should affect your revision plans and exam tactics (Chapter 18), taking into account (a) any choices or restrictions offered in the paper, and (b) the different types of questions asked (i.e. multiple choice, short-answer or essay).
- 4. Examine carefully the style of questions.** Can you identify the expectations of your lecturers? Can you relate the questions to the learning objectives? How much extra reading is expected? Are the questions fact-based? Do they require analysis or synthesis based on other knowledge? Consider how the answers to these questions might affect your revision effort and exam strategy.
- 5. Practise answering questions.** Perhaps with friends, set up your own mock exam when you have done a fair amount of revision, but not too close to the exams. Use a relevant past exam paper; and try not to study it beforehand. You need not attempt all of the paper at one sitting. You will require a quiet room in a place where you will not be interrupted. Keep close track of time during the mock exam and try to do each question in the length of time you would normally assign to it (see p. 76) – this gives you a feel for the speed of thought and writing required and the scope of answer possible. Mark each other's papers and discuss how each of you interpreted the question and laid out your answers and your individual marking schemes. Remember that this type of self-testing can enhance your learning (p. 19), enabling you to work on any aspects that you self-identify as being weak.
- 6. Practise writing answer plans and starting answers.** This can save time compared with the 'mock exam' approach. Practise in starting answers can help you get over stalling at the start and wasting valuable time. Planning and writing exam answers as 'bullet point' summaries gets you used to organising your thoughts quickly and putting your thoughts into a logical sequence.

Recognising when your concentration powers are dwindling – take a short break from revision when this happens and return to work refreshed and ready to learn. Remember that 20 minutes is often quoted as a typical limit to a period of full concentration (p. 19).

Question-spotting – avoid this risky strategy. You may find that you are unable to answer any unexpected topics that you failed to revise. Moreover, if you have a preconceived idea about what will be asked, you may also fail to grasp the nuances of the exact question set, and provide a response lacking in relevance.

Tackling the revision process

Begin early, to avoid last-minute panic. Start several weeks before the exam, and plan your work carefully:

- **Prepare a revision timetable** – an 'action plan' that gives details of specific topics to be covered (Box 17.2). Plan your revision timetable around the dates/times of each exam and stick to it. Time-management during this period is as important as keeping to time during the exam itself.
- **Study the learning objectives/outcomes for each topic** to get an idea of what lecturers expect from you (p. 18).
- **Use past papers as a guide** to the form of exam and the type of question likely to be asked (Box 17.1).
- **Remember to have several short (five-minute) breaks during each hour of revision and a longer break every few hours.** In any day, try to work for a maximum of around three-quarters of the time.
- **Include recreation within your schedule:** there is little point in tiring yourself with too much revision – you will learn more in shorter, focused sessions, with spacing.

Box 17.2 How to prepare and use a revision timetable

This can be prepared in digital format (e.g. using Microsoft Office templates) or using paper and pen, as in the following description.

- 1. Make up a grid showing the number of days until your exams are finished.** Divide each day into several sections. It is best to work in short periods, each devoted to a specific topic – for example, one-hour periods.
- 2. Write in your non-revision commitments**, including any time you plan to allocate to physical activity. Try to have about one-third or a quarter of the time off in any one day. Plan this in relation to your best times for useful work – for example, some people feel they work best in the mornings, while others prefer evenings. If you wish, use a system where your relaxation time is a bonus to be worked for; this may help you motivate yourself.
- 3. Decide on how you wish to subdivide your subjects** for revision purposes. This might be among subjects, according to difficulty (with the hardest getting the most time), or within subjects, according to topics. Make sure there is an adequate balance of time among topics and especially that you do not avoid working on the subject(s) you find least interesting or most difficult.
- 4. Allocate the work to the different slots available on your timetable.** You may wish to colour-code the subjects. You should work backwards from the exams, making sure that you cover every exam topic adequately in the period immediately before each exam. Remember the learning value of spacing and retrieving/reviewing (Box 3.1) each topic during the revision period.
- 5. As you revise, mark off the slots completed** – this has a positive psychological effect and will boost your self-confidence.
- 6. After the exams, revisit your timetable** and decide whether you would prepare differently next time.

Box 17.3 How to revise actively

The following techniques may prove useful in devising an active revision strategy:

- **Aid recall through effective note-making:** for example, the Mind Map technique (p. 27) is claimed to enhance recall for some by connecting the material to visual images.
- **'Distil' your lecture notes** to show the main headings and examples. Prepare revision sheets with details for a particular topic on a single sheet of paper, arranged as a numbered checklist. Wall posters are another useful revision aid.
- **Make up lists of key facts and definitions:** these can be a useful starting point for many exam answers. Try to remember *how many* points you need to know in each case – this might help you recall them all during the exam.
- **Write key points on 'Post-it®' notes or similar:** arrange and rearrange these in clusters or as lists around your study space or room.
- **Use mnemonics and acronyms** to commit specific factual information to memory. Sometimes, the dafter they are, the better they seem to stick in your memory.
- **Draw diagrams from memory:** make sure you can label them fully.
- **Try recitation as an alternative to written recall.** Talk about your topic to another person, preferably someone in your class, or to yourself. Explaining something out loud is an excellent test of your understanding.
- **Associate facts with images or journeys** if you find this method works.
- **Use a wide variety of approaches** to avoid boredom during revision (e.g. record information on your smartphone's voice recorder, use cartoons, or any other method, as long as it is not just reading).
- **Use frequent self-assessment during your revision:** this will show whether you have retained the information you are revising. Assess what you have learned by taking a blank sheet of paper and writing down all you know on a selected topic. Check your full notes to see if you missed anything out – this works best if you are trying to recall a specific number of items from a checklist. If anything was missed, go back immediately to a fresh blank sheet and redo the example. Repeat, as required.
- **Form a revision group** to share ideas and discuss topics with other students.
- **Prepare answers to past papers**, e.g. write essay plans and outlines (see Box 17.1).
- **If your subject involves numerical calculations, work through representative problems.** Past papers may include appropriate examples.
- **Make up your own questions:** the act of putting yourself in the examiner's mindset by inventing and answering your own questions can help your revision. Software such as PeerWise (see: <https://peerwise.cs.auckland.ac.nz/>) can help you work collaboratively on exam questions and answers.

- **Make your revision as active as possible** (see Box 17.3): the least productive approach is simply to read and reread your notes. Answering mock questions and completing past papers not only tests your *memory* of factual information, but also your *understanding* of how to use/apply what you have learned.
- **Ease back on the revision near the exam:** plan your revision to avoid last-minute cramming and overload fatigue.

KEY POINT When answering exam questions, look carefully at words used in the instructions, as they can help you identify what depth is expected in your answer (see Tables 3.1 and 11.1). Take special care in multi-part questions, because the first part may require lower-level thinking, while deeper thinking may be required in subsequent parts.

Making final preparations – try to get a good night's sleep before an exam (Box 3.1). Last-minute cramming will be counter-productive if you are too tired during the exam.

The evening before your exam should be spent in consolidating material, and self-testing of summary lists, key facts and definitions. Avoid introducing new material at this stage: your aim should be to boost your confidence, putting yourself in the right frame of mind for the exam itself.

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[Many universities host study skills websites; these can be found using 'study skills', 'revision' or 'exams' as key words in a search engine. Your library may also hold useful information, for example: <http://libguides.reading.ac.uk/exams/revision>. Last accessed 28/04/21]

STUDY EXERCISES

17.1 Make use of past exam papers. Use the techniques discussed in Box 17.1 to improve your revision strategy.

17.2 Draw up a revision timetable. Use the techniques discussed in Box 17.2 to create a revision timetable for your forthcoming exams. You may wish to use or adapt the arrangement below, either on paper or within a spreadsheet.

A revision timetable planner

Date	Morning		Lunch	Afternoon		Tea/Dinner	Evening	
	Session 1	Session 2		Session 1	Session 2		Session 1	Session 2

Answers to these study exercises are available at go.pearson.com/uk/he/resources

18 Improving your performance in exams

Definitions

Some terms are used with specialised meaning in university exam contexts.

Diet – a period during which a number of exams take place, according to a published timetable.

Invigilator – a person whose role is to ensure an exam is conducted according to the regulations.

Paper – usually, a set of questions for a single subject.

Question – an element of an exam paper, often couched in terms of an instruction rather than a true question, and often with the marks available stated at the end.

Rubric – the set of instructions at the start of an exam paper.

Subject representative – a staff member who knows about the exam topic and who can sort out any issues with the format or question wording (often only present at the start of the exam).

You are unlikely to have reached this stage in your education without being exposed to the examination process. While you may not always enjoy this form of assessment, you will want to do as well as possible in your course. The advice in this chapter assumes that you have prepared well through effective revision (Chapter 17). It focusses on ‘high-stakes’ assessment conducted under exam conditions; other assessments may contribute to your overall mark and advice for these is distributed throughout this book.

KEY POINT To succeed in exams, it is essential to understand why and how you are being tested (Chapter 9) so you can arrive at an appropriate strategy for each exam and for the individual style(s) of questions used in the exam.

Preparing for the exam

You should have researched the location, timing and format of the exam during your revision planning (Chapter 17). Using the results, you should create a plan for tackling the exam. This should ensure the proportion of time you allocate for each component is roughly in proportion to the marks that can be gained, that you provide some time for reading the questions and planning your answers (if required) and leave some time for checking through your answers.

On the day of the exam, give yourself sufficient time to arrive at the correct room, without the risk of being late. If you arrive early, you can always set right a mistake if you find you have gone to the wrong place. As part of your preparation, make a checklist of the items you will need to bring to the exam (Box 18.1). Knowing that you have prepared well, checked everything on your list and gathered together all you need for an exam will improve your confidence and reduce anxiety.

Box 18.1 How to create an exam action list

Relevant actions might include:

- Confirming the time, date and place of the exam.
- Planning your travel arrangements to exam hall.
- Double-checking module handbooks and past papers for exam structure.
- Thinking through your use of time and your exam strategy.
- Identifying a quiet place near the exam hall to carry out a last-minute check on key knowledge (e.g. formulae, definitions, diagram labels).
- Ensuring you have all the items you wish to take to the exam, e.g.
 - pens, pencils (with sharpener and eraser)
 - laptop or tablet (fully charged), where relevant
 - ruler
 - correction fluid
 - calculator (allowable type)
 - sweets and drink, if allowed
 - tissues
 - watch or clock
 - ID card
 - texts and/or notes, if an open-book exam
 - mascot.
- Setting alarm(s) and/or asking a friend or family member to check you are awake on time.

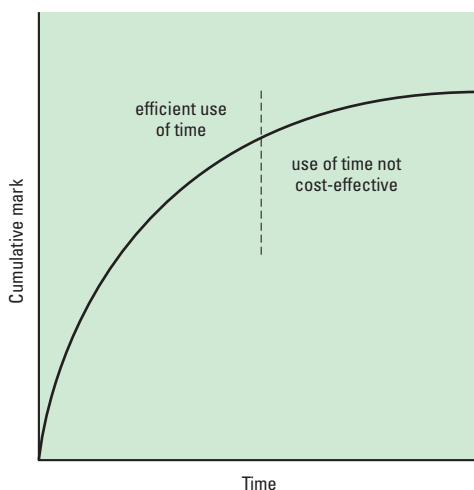


Fig. 18.1 Potential exam marks as a function of time. The marks awarded in a single answer will follow the law of diminishing returns – it will be far more difficult to achieve the final 25% of the available marks than the initial 25%. This shows why you should not spend too long on any one question: you might gain more marks by devoting the ‘extra’ time to another question.

Using the question paper – unless this is specifically forbidden, you *should* write on the question paper to plan your strategy, keep to time and organise your answers.

Focussing on the substance – try to get into the ‘meat’ of your essay answer as quickly as you can – don’t spend too long refining your introductory paragraphs.

Tackling the paper

Work in a focussed and determined way as soon as the exam begins. Begin by reading the instructions at the top of the exam paper (the ‘rubric’) carefully and confirm that it is as you expected, so that you do not make any errors based on lack of understanding of the exam structure. If it helps, underline or highlight the key phrases in these instructions, to reinforce their message. Check whether different questions should be answered in different books.

Take the first few minutes to read the paper and refine your strategy, before you begin writing. Do not be put off by those who begin immediately; it is almost certain they are producing unplanned work of a poor standard. Assess the set of questions, focussing on their wording and relationship to the revision you have done. Where there is a choice, decide on those questions you wish to answer and the order in which you will tackle them. Use the exam paper to mark the sequence in which the questions will be answered, and write the anticipated finishing times alongside; refer to this timetable during the exam to keep yourself on course. Stick with your plan as far as possible. In particular, do not be tempted to spend too long on any one question or section just because you know a lot about the topic: the return in terms of marks will not justify the loss of time from other questions (see Fig. 18.1).

Taking account of different types of exam question

Essay questions

Essay questions let examiners test the depth of your comprehension and understanding as well as your recall of facts. Essay questions give you plenty of scope to show what you know.

Before you tackle a particular essay question, you must be sure of what is required in your answer. Ask yourself ‘What is the examiner looking for in this particular question?’ and then set about providing a *relevant* answer. Consider each individual word in the question and highlight, underline or circle the key words. Make sure you know the meaning of the terms given in Table 11.1 so that you can provide the appropriate information, where necessary. Spend a little time thinking through a structure for your writing (see Chapter 11). Refer back to the question frequently as you write, to confirm that you are keeping to the subject matter: Box 18.2 gives further advice on writing essays under exam conditions.

It is usually a good idea to begin with the question that you are most confident about. ‘Banking’ a reasonably good answer will reassure you before tackling more difficult parts of the paper. Try to follow the guidelines for providing a strong essay-style answer (Box 18.3). If you run out of time, write your answer in note form. Examiners may be relatively sympathetic to this as long as the main components of the question have been addressed and the intended structure of the answer is clear.

Multiple-choice and short-answer questions

Multiple-choice questions (MCQs) and short-answer questions (SAQs) are generally used to test the breadth and detail of your knowledge. The various styles that can be encompassed within the SAQ format allow for more demanding questions than MCQs, which may emphasise specific factual knowledge.

Box 18.2 How to write under exam conditions

Always go into an exam with a strategy for managing the available time.

- **Allocate some time (say 5% of the total) to consider which questions to answer and in which order.**
- **Share the rest of the time among the questions, according to the marks available.** Aim to optimise the marks obtained. A potentially good answer should be allocated slightly more time than one you do not feel so happy about. However, do not concentrate on any one answer, as this is counter-productive (see Fig. 18.1).
- **For each question divide the time into planning, writing and revision phases** (see p. 76). Employ time-saving techniques as much as possible.
- **Use spider diagrams** (Fig. 10.2) **or Mind Maps** (Fig. 4.3) to organise and plan your answer.
- **Use diagrams and tables** to save time in making difficult and lengthy explanations, but make sure you refer to each one in the text.
- **Use standard abbreviations for the subject** to save time repeating text but always explain them at the first point of use.
- **Consider speed of writing and neatness** – especially when selecting the type of pen to use – ballpoint pens are fastest, but they tend to smudge. You can only gain marks if the examiner can read your script.
- **Keep your answer simple and to the point**, with clear explanations of your reasoning. Make sure your answer is relevant.
- **Do not include irrelevant facts** just because you memorised them during revision, as this may do you more harm than good. You must answer the specific question that has been set. *Remember that time taken to write irrelevant material is time lost from another question.*

Box 18.3 How to provide strong exam answers to essay-style questions

- **Answer the exact question set.** Recognise the specialist terms used in the question, and demonstrate an understanding of the terms by providing definitions. Carry out the precise instruction in the question, ensuring you address all relevant aspects.
 - **Keep to time.** Try your best to stick your predetermined timetable. Match the time allocated to the marks allocated to the answer. Avoid spending too long on one question and not enough on the others, or even failing to complete the paper.
 - **Answer all parts of a multiple-part question.** Recognise when one part (perhaps involving more complex ideas) may carry more marks than another.
 - **Provide evidence to support your answer.** Remember to 'state the obvious' – as this applies to either basic facts or definitions (there will be notional marks allocated to these).
 - **Illustrate your answer appropriately.** Including a relevant diagram, ensuring this genuinely aids communication. Make sure the examples you quote are fully relevant.
 - **Give a complete, relevant, answer.** Ensure all *relevant* knowledge is used in your answer. Do not include irrelevant evidence to support an answer or 'waffle' to fill space.
 - **Make sure your handwriting is legible.**
 - **Ensure the facts and ideas you mention are expressed clearly and in good English.**
 - **Make sure there is a structure and logic to your answer.**
 - **Try to ensure there are no factual errors and correct obvious mistakes** by rereading your answer before submitting the script.
- At higher levels, the following aspects are especially important:
- **Provide enough in-depth information.**
 - **Provide an analytical rather than a descriptive answer.** Focus on the 'deeper' aspects of a topic rather than bare facts (Table 3.1 provides additional guidance).
 - **Set problems in context to demonstrate your wider understanding of the topic** – Make sure you do not overdo this, or you may risk not answering the question set.
 - **Give evidence of reading around the subject.** This can be demonstrated, for example, by quoting relevant papers and reviews and by giving author names and dates of publication.
 - **Considering both sides of a topic/debate.**
 - **Arrive at a conclusion** – especially if you have been specifically asked to do so.

Guessing answers – if there is a penalty for incorrect answers in a multiple-choice test, the best strategy is not to answer questions when you know your answer is a complete guess. Depending on the penalty, it may be beneficial to guess if you can narrow the choice down to two options. However, if there are no such penalties, then you should provide an answer to all questions. Finding out what the rules are should be part of your exam preparation.

Answering the question as requested – this is true for all questions, but especially important for SAQs. If the question asks for *n* aspects of a topic, try to list this number of points; if there are two or more parts, provide appropriate answers to all aspects; if the question asks for a diagram, make sure you provide one. This may seem obvious, but many marks are lost for not following instructions.

A good approach for MCQ papers is as follows:

1. **First trawl:** read through all the questions fairly rapidly, noting the ‘correct’ answer for those you can attempt immediately.
2. **Second trawl:** go through the question set again, now dealing with the straightforward but more difficult questions, but leaving those that require a lot of thought or time.
3. **Third trawl:** now tackle the most difficult questions and those that require longer to answer (for example, those based on numerical problems).
4. **Final review:** look again at all your answers, checking for obvious mistakes.

This approach is not only efficient, but it also gives scope for other questions to prompt you to recall forgotten facts (which happens surprisingly frequently).

When unsure of an answer, the first stage is to rule out options that are clearly absurd or have obviously been placed there to distract you. Next, looking at the remaining options, can you judge between contrasting pairs with alternative answers? Logically, both cannot be correct, so you should see if you can rule one of the pair out. Watch out, however, in case *both* may be irrelevant to the answer. If the question involves a calculation, try to work this out independently from the answers, so you are not influenced by them.

In SAQ papers, there may be a choice of questions. A careful reading is essential to grasp all the nuances of each one. Choose your options carefully – it may be better to gain half marks for a correct answer to half a question, than to provide a largely irrelevant answer that lacks the necessary focus and detail. For this form of question, few if any marks are given for writing style. Think in ‘bullet point’ mode and list the crucial points only. The time for answering SAQs may be tight, so get down to work fast, starting with answers that demand remembered facts. Stick to your timetable by moving on to the next question as soon as possible. Strategically, it is probably better to get part-marks for the full number of questions than good marks for only a few.

Extended matching items (EMI) questions

These are a more complex and challenging version of the multiple-choice format, where a pool of answers is offered that may be correct in several cases or none at all. They may also be called extended matching sets or extended matching questions. Formats differ, but the questions often relate to a complex scenario outlined as a ‘vignette’. EMIs are designed to test not only your detailed knowledge but also your deeper understanding and ability to apply this. They are commonly used in disciplines where professional judgements on complex issues must be made.

Exams with an EMI component require you to read carefully, quickly and with understanding, distinguish between relevant and irrelevant information and make accurate connections between statements. As with MCQs, you will require a broad knowledge across your subject, but also a deeper and sometimes quite detailed understanding. An idea of the sorts of topics that might be covered should be apparent from the learning outcomes and case studies covered in the course. Look carefully at any example EMIs

Example Typical layout for an extended matching items (EMI) question:

1. **Description of topic or theme** – use this heading to orientate your thoughts when answering.
2. **Instructions** – this part will explain how you should answer and the weighting of marks among the questions. If all the questions are similar, this may appear at the head of the exam paper; if not, read each instruction carefully, as the rules and marks given may change.
3. **A series of options** – you will have to select among these to answer the questions which follow. Some options may be correct more than once, some not at all, and you may or may not be told how many are correct in each case. Each time you answer a question you will have to go through the list carefully and see which apply – slow readers beware!
4. **A scenario (or 'vignette')** – this is a short paragraph that describes the background to the questions. Details here will have a big effect on the answers you give, so you will need to consider the wording very carefully before you answer. It might be a good idea to highlight key words to keep them in your mind.
5. **The questions** – these may be grouped, and may develop in complexity from those demanding a simple factual answer to those requiring a fair amount of reflection and judgement.

or past papers that are offered, as this will help you develop a strategy for answering.

When answering an EMI paper, first look quickly through the topics and select those that most suit your knowledge and revision. Plan to answer those you are most confident about first. Then, for each individual EMI:

1. **Scan-read the whole question quickly** to get a feel for its scope.
2. **Carefully read both the scenario and the questions a second time**, and highlight key terms. Some people find it helpful next to try to answer the questions without reference to the options as this avoids the chance of being put off by distractors.
3. **Now run through the list of options ruling them 'in' or 'out'** for each specific question – you should be left with a short list of possible answers.
4. **Select the final answers you wish to give**, with reference to the instructions or rules provided.
5. **Make sure you are keeping up with the necessary pace** by setting a target time for completion of each topic. Try to leave some time in your planning to allow you to review and check your answers (p. 112).

You will need to be particularly well focused and work quickly and decisively – time will often be a constraint in EMI exams. If there are no penalty marks, ensure you give a response to each question.

Practical and information-processing exams

The prospect of a practical or information-processing exam in biomolecular science may cause you more concern than a theory exam. This may be due to a limited experience of practical examinations, or to the fact that practical and observational skills are tested, as well as recall, description and analysis of factual information. Your first thoughts may be that it is not possible to prepare for such exams but, in fact, you can improve your performance by mastering the various practical techniques described in this book.

You may be allowed to take your laboratory reports and other texts into the practical exam. Do not assume that this is an easy option, or that revision is unnecessary: you will not have time to read large sections of your reports or to familiarise yourself with basic principles, etc. The main advantage of 'open book' exams is that you can check specific details of methodology, reducing your reliance on memory, provided you know your way around your practical manual. In all other respects, your revision and preparation for such exams should be similar to theory exams. Make sure you are familiar with *all* of the practical exercises, including any work carried out in class by your partner (since exams are assessed on individual performance). If necessary, check with the teaching staff to see whether you can be given access to the laboratory, to complete any exercises that you have missed.

At the outset of the practical exam, determine or decide on the order in which you will tackle the questions. A question in the latter half of the paper may need to be started early on in the exam period (for example, an enzyme assay requiring 2-h incubation in a 3-h exam). Such questions are included to test your forward-planning and time-management skills. You may need to make additional decisions on the allocation of material, for example, if you are given 30 sterile test tubes, there is little value in designing an experiment that uses 25 of these to answer question 1, only to find that you need at least 15 tubes for subsequent questions.

Examples These are the principal types of question you are likely to encounter in a practical or information-processing exam:

Calculations

For example, the preparation of aqueous solutions at particular concentrations (Chapter 23), or numerical/statistical problems (Chapters 75–77). Tests numeracy.

Data analyses

May include the preparation and interpretation of graphs (Chapter 74) or data sets, from information obtained during the exam or provided by the examiner. Tests problem-solving skills.

Drawing specimens

Accurate representation and labelling will be important. Tests drawing and interpretation abilities.

Interpreting images

Sometimes used when it is not possible to provide living specimens, e.g. in relation to fieldwork, or electron microscopy. Can test a variety of skills.

Manipulative exercises

Often based on work carried out during your practical course. Tests dexterity, specific techniques (e.g. sterile technique, Chapter 33).

Preparing specimens for examination with a microscope

Tests staining technique and light microscopy technique (Chapter 26).

'Spot' tests

Short questions requiring an identification, or brief descriptive notes on a specific item (e.g. a prepared slide). Tests knowledge of seen material or ability to transfer this to a new example.

Understanding terminology – borrowing Latin, an oral exam is sometimes known as a 'viva voce' (by or with the living voice) or 'viva'.

Make sure you explain your choice of apparatus and experimental design. Calculations should be set out in a stepwise manner, so that credit can be given, even if the final answer is incorrect (see p. 587). If there are any questions that rely on recall of factual information and you are unable to remember specific details; for example, if you cannot identify a particular specimen or slide, make sure that you describe the item fully, so that you gain credit for observational skills. Alternatively, leave a gap and return to the question at a later stage.

Oral exams and interviews

An oral interview is sometimes a part of final degree exams, representing a chance for the external examiner(s) to get to know the students personally and to test their abilities directly and interactively. In some departments, orals are used to validate the exam standard, or to test students on the borderline between exam grades. Sometimes an interview may form part of an assessment, as with project work or posters. This type of exam is often intimidating – many students say they do not know how to revise for an oral – and many candidates worry that they will be so nervous they will not be able to do themselves justice.

Preparation is just as important for orals as it is for written exams:

- **Think about your earlier performances** – if the oral follows written papers, it may be that you will be asked about questions you did not do so well on. These topics should be revised thoroughly. Be prepared to say how you would approach the questions if given a second chance.
- **Read up a little about the examiner** – he or she may focus their questions in their area of expertise.
- **Get used to giving spoken answers** – it is often difficult to transfer between written and spoken modes. Write down a few questions and get a friend to ask you them, possibly with unscripted follow-up queries.
- **Research and think about topical issues in your subject area** – some examiners will feel this reflects the depth of your understanding and knowledge and how interested you are in your subject.

Your conduct during the oral exam is important too:

- **Arrive promptly and wear reasonably smart clothing.** Not to do either might be considered disrespectful by the examiner.
- **Take your time before answering questions.** Even if you think you know the answer immediately, take a while to check mentally whether you have considered all angles. A considered, logical approach will be more impressive than a quick but ill-considered response.
- **Start answers with the basics, then develop into deeper aspects.** There may be both surface and deeper aspects to a topic and more credit will be given to students who put the latter in context.
- **When your answer is finished, stop speaking.** A short, crisp answer is better than a rambling one.
- **If you do not know the answer, say so.** To waffle and talk about irrelevant material is more damaging than admitting that you do not know. In some cases, the examiner may provide a hint designed to jog your memory.

Allowing yourself to relax in an oral exam – external examiners are generally experienced at putting students at ease. They will start by asking ‘simple-to-answer’ questions, such as what modules you did, how your project research went and what your career aspirations are. Imagine the external examiner as a friend rather than a foe.

- **Make sure your answer is balanced.** Talk about the evidence and opinions on both sides of a contentious issue.
- **Do not disagree violently with the examiner.** Politely put your point of view, detailing the evidence behind it. Examiners will be impressed by students who know their own mind and subject area. However, they will expect you to support a position at odds with the conventional viewpoint.
- **Above all, be positive and enthusiastic about your topic.**

Reviewing your answers

At the end of the exam, you should allow some time to read through your script or to review multiple choice answers, to check for:

- **errors of fact**
- **missing information**
- **grammatical and spelling errors**
- **errors of scale and units**
- **errors in calculations.**

Make sure your name and/or ID number is on each exam book as required and on all other sheets of paper, including graph paper, even if securely attached to your script, as it is in your interest to ensure that your work does not go astray. For computer-based exams, complete all necessary registration details.

KEY POINT *Never leave any exam early. Most exams assess work carried out over several months in a time period of 2–3 hours and there is always something constructive you can do with the remaining time to improve your script. In essay answers, for example, it is generally acceptable to add inserts and refer to these by numbers or arrows.*

Counteracting anxiety before and during exams

Adverse effects of anxiety need to be overcome by anticipation and preparation well in advance (Box 18.4). Exams, with their tight time limits, are especially stressful for perfectionists. To counteract this tendency, focus on the following points during the exam:

- **Accept a less-than-perfect answer** – excellence comes at a cost in time that might be better spent on other questions.
- **Only spend a short time planning your answer** – once you have an outline plan, get started.
- **Make sure you cover the main message** – rather than spending too much time on the initial parts of an answer.
- **Concentrate on getting all of the basic points across** – examiners usually reserve some marks for the main points, before allocating extra marks for detail and depth.
- **Make sure your answers are legible** – but do not be obsessed with neatness, either in handwriting, or in the diagrams you draw.

Box 18.4 How to deal with symptoms of exam anxiety

- **Sleeplessness** – this is commonplace and does little harm in the short term. Get up, have a snack, do some light reading or other activity, then return to bed. Avoid caffeine (e.g. tea, coffee and cola) for several hours before going to bed.
- **Lack of appetite** – again commonplace. Eat what you can, but take sugary sweets into the exam to keep energy levels up in case you become tired.
- **Fear of the unknown** – it can be a good idea to visit the exam room beforehand, so you can become familiar with the location. By working through the points given in the exam action list on p. 117 you will be confident that nothing has been left out.
- **Worries about timekeeping** – double-check your alarm is functioning and use more than one system if possible. Ask a friend or relative to make sure you are awake on time. Make reliable travel arrangements, to arrive on time. If your exam is early in the morning, it may be a good idea to get up early for a few days beforehand.
- **Blind panic during an exam** – explain how you feel to an invigilator. Ask to go for a supervised walk outside. Do some relaxation exercises (see below), then return to your work. If you are having problems with a specific question, it may be appropriate to speak to the departmental representative at the exam.
- **Feeling tense** – shut your eyes, take several slow, deep breaths, do some stretching and relaxing muscle movements. During lengthy exams, it may be a good idea to do this between questions, and possibly to have a complete rest for half a minute or so. Prior to exams, try some exercise activity or escape temporarily from your worries by watching TV or a movie.
- **Feeling you are running out of time** – do not panic when the invigilator says 'five minutes left'. Just try to speed up. It is amazing how much you can write in this time. Write note-style answers or state the areas you would have covered: you may get some credit.
- **Thinking you are underprepared** – the remedy, of course, is to adopt an effective approach to your planning and revision.

Discussing the exam – avoid becoming involved in prolonged analyses with other students over the 'ideal' answers to the questions; after all, it is too late to change anything at this stage. Go for a walk, watch TV for a while, or do something else that helps you relax, so that you are ready to face the next exam with confidence.

- **You cannot be expected to know everything about the topic** – do not worry if you forget something. Most examiners will give more credit to answers that show an understanding of the bigger picture, rather than finer detail such as the year a particular paper was published. Remember, many marking schemes give a first-class grade to work that misses out on up to 30% of the marks available.

KEY POINT Everyone worries about exams. Anxiety is a perfectly natural feeling. It works to your advantage, as it helps provide motivation and the adrenaline that can help you 'raise your game' on the day.

There is a lot to be said for tackling exams as you would a game. After all, they are artificial situations contrived to ensure that large numbers of candidates can be assessed together, with little risk of cheating. They have conventions and rules, just like games. If you understand the rationale behind them and follow the rules, this will aid your performance.

Sources for further study

Anon. *Preparing for exams. Expert guidance from Study Advice at the University of Reading*. Available: <https://libguides.reading.ac.uk/exams/international>
Last accessed 24/04/21.

[Generic guidance on university exams from the University of Reading, UK. A downloadable pdf file is available. Your university may host similar Web pages.]

McMillan, K.M. (2021) *The Study Skills Book*, 4th edn. Pearson Education, London.

McMillan, K.W. and Weyers, J.D.B. (2011) *How to Succeed in Exams and Assessments*. Pearson Education, London.

O'Brien, D. (2007) *How to Pass Exams: Accelerate Your Learning – Memorise Key Facts – Revise Effectively*, 2nd edn. Duncan Baird Publishers, Winchester.

[Many universities host study skills websites that cover exam technique; these can be found using 'study skills', 'revision' or 'exams' as key words in a search engine.]

STUDY EXERCISES

18.1 Analyse your past performances. Think back to past exams and any feedback you received from them. How might you improve your performance? Consider ways in which you might approach the forthcoming exam differently. If you have kept past papers and answers to continuous assessment exercises, look at any specific comments your lecturers may have made.

18.2 Share revision notes with other students. Make a revision plan (see p. 108) and then allocate some time

to discussing your revision notes with a classmate. Try to learn from their approach. Discuss any issues you do not agree upon.

18.3 Plan your exam tactics. Find out from published guidance or past papers what the format of each paper will be. Confirm this with staff. Decide how you will tackle each paper, allocating time to each section and to each question within the sections (see p. 112). Write a personal checklist of requirements for the exam (see p. 116).

Answers to these study exercises are available at go.pearson.com/uk/he/resources



Fundamental laboratory techniques

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19 Preparing for practical work

Developing your practical skills – this will involve:

- carrying out 'standard' procedures
- designing experiments
- observing and measuring
- recording data
- analysing and interpreting data
- reporting/presenting.

Preparing for fieldwork – in some cases you may need to collect samples from the field, or field excursions might be a part of your course. If this is the case, make sure you wear clothing and footwear appropriate to the expected weather and terrain.

Using textbooks in the lab – where appropriate, take this book, or photocopies of selected chapters, along to the relevant classes, so that you can make full use of the relevant information during the practical sessions.

All knowledge and theory in science arises from practical observation and experimentation: this is equally true for disciplines as diverse as microscopy and molecular genetics. Practical work is an important part of most courses and often accounts for a significant proportion of the assessment marks. The 'hands-on' skills and abilities that you develop in practical classes and project work will continue to be useful throughout your course and beyond, some within science and others in any career you choose (see Chapter 1).

Making the most of practical work

To optimise learning from lab classes, you should:

- **Prepare as well as you can by reading through the schedule in advance** – make sure you understand the purpose of the practical and the particular skills involved. Does the practical relate to, or expand upon, a current topic in your lectures? Is there any additional preparatory reading that will help?
- **Make sure you have all the equipment and any specific clothing you may need** – for lab sessions, this will include a clean lab coat and safety glasses (Fig. 20.1), and in some cases additional personal protective equipment (PPE).
- **Consider what safety hazards might be involved** – think about what precautions you might need to take, before you begin the session (p. 124).
- **Consider potential ethical issues at an early stage** – these are discussed in Chapters 30 and 32.
- **Arrive at the lab or assembly point in good time** – if you are late, you may miss important points delivered before practical work begins, as well as putting yourself under time pressure to complete the session.
- **Listen carefully to any introductory guidance provided by the lecturer in charge, noting any important points** – adjust your schedule/handout as necessary.
- **Organise your laboratory bench space before starting to work** – make sure your lab notebook, calculator and pen/pencil are adjacent to your working area. Collect or assemble all the items you will require.
- **Keep your bench space tidy throughout** – you will often find it easiest to keep clean items of glassware, etc. on one side of your working space, with used equipment on the other side. Follow safe working procedures at all times.
- **Ask questions whenever you do not understand** – staff and postgraduate demonstrators are there to help you, so ensure you make the most of their knowledge by listening to their advice and asking them questions. However, do not expect them to hand you the answers on a plate – they will probably be looking to make you think for yourself, and may only provide helpful suggestions once they hear your ideas.
- **Work speedily during the sessions** – use time when waiting for something to happen to start your write-up.
- **Create meaningful notes (p. 18)** – ensure that you will be able to decipher and interpret these when you write up the session.

SAFETY NOTE Smartphones should never be used in a lab class, as there is a risk of contamination from hazardous substances. Always switch off your mobile phone before entering a laboratory.

- **Prepare tables and draw up informal figures as you go along** – Chapters 73 and 74 give specific advice.
- **Complete your write-up as soon as possible after the event** – this way, you will reinforce your learning (p. 215) and you will also avoid losing marks.
- **Catch up on any work you have missed as soon as possible** – preferably, before the next session, since subsequent practicals often build on material and techniques developed in previous sessions.

KEY POINT You will get the most out of practicals if you prepare in advance. Approach practical sessions with a positive, inquiring mindset and don't assume that you can take a passive role, where everything will be provided – you will need to engage with the activities, follow any standard procedures and think about the meaning of results.

Getting to grips with bioethics – in addition to any moral implications of your lab practicals and fieldwork, you may have the opportunity to address broader issues within your course (see Chapter 30). Professional scientists must always consider the consequences of their work, and it is therefore important that you develop your understanding of these issues alongside your academic studies.

Following ethical, safety and legal requirements

You will need to consider these issues at all times during your practical work. Ensure that you:

- **Consider the ethical aspects of laboratory work that involves human or animal subjects carefully.** Chapter 30 discusses this in more depth.
- **Follow safe working practice, in line with relevant codes and legislation.** There is also a moral obligation to avoid harm to yourself and others; this is covered in more detail in Chapter 20.

Considering what you need for practical classes

Personal protective equipment (PPE)

You will need two lab coats (one to replace the other while it is being laundered) – only wear your lab coat during classes (outdoor coats and bags need to be stored well away from lab benches, to avoid contamination). Ensure your lab coat is buttoned up during practical procedures, to protect the front of your body. Similarly, you will need a pair of safety glasses with side shields – check whether you need to buy your own, or whether these are provided for you, when required. Always wear closed-in footwear in lab classes. You may need to wear additional items, such as gloves or goggles, depending on the risk assessment (p. 125) for individual practical sessions.

Equipment for recording your observations and results

When you start your degree studies, you are likely to be provided with printed schedules to guide you through individual lab sessions. This may include an introduction to the practical; instructions for handling samples, making observations, following procedures, or using instruments/materials; details of any experiments to be designed and/or carried out; and, in some cases, spaces for drawing diagrams, recording your results and making notes. The schedule may also include 'prompts' for analysis or conclusions, sometimes in the form of questions, with spaces for answers.

Presenting your results – the format and layout you use are important aspects, since you will gain marks for well-presented work. Chapters 73 and 74 give further practical advice for results presented in tabular and graphical formats.

In later years, you will be expected to organise your own laboratory notes. An A4 loose-leaf ring binder offers flexibility, since you can insert laboratory handouts or lined and graph paper at appropriate points. The danger of losing one or more pages from a loose-leaf system is the main drawback. Bound notebooks avoid this problem, although those containing alternating lined/graph or lined/blank pages tend to be wasteful – it is often better to paste sheets of graph paper into a bound book, as required.

Good-quality pencils (plus an eraser and sharpener) are recommended for recording your raw data, making diagrams, etc., as mistakes are easily corrected. Buy a black, spirit-based (permanent) marker for labelling experimental glassware, Petri plates, etc. Fine-tipped drawing pens are useful for preparing hand-drawn final versions of graphs and diagrams for assessment. Use a see-through ruler with an undamaged edge for hand-drawn graphs, so that you can see data points and information underneath the ruler as you draw. Alternatively, use graph drawing software such as Microsoft *Excel* (see Chapter 74).

Using inexpensive calculators – take care, as some unsophisticated calculators have a restricted display for exponential numbers and do not show the ‘power of 10’, e.g. displaying 2.4×10^{-5} as 2.4^{-05} , or even $2.4E - 05$, or $2.4 - 05$.

Choosing and using a calculator

In the early stages of your studies, it is usually better to avoid complex calculators with impressive-looking but often unused pre-programmed functions. Opt for basic mathematical functions (logarithms, roots), parentheses (brackets) and statistical functions (means and standard deviations, Chapter 76) rather than engineering constants. Programmable calculators can be useful for more advanced studies, but note that they are usually unacceptable for exam use. Many university departments specify a particular make and model of calculator for students to use from the first year onwards. The advantage of this approach is that you can seek help from staff and demonstrators during tutorials and lab classes, if you are unsure of a specific function. Conversely, teaching staff may not be fully familiar with other types of calculator and may not be able to answer such questions if you are using an alternative make or model.

Using calculators for numerical problems – Chapter 75 gives further advice on tackling numerical problems.

Source for further study

Barnard, C.J., Gilbert, F.S. and MacGregor, P.K. (2011) *Asking Questions in Biology*, 4th edn. Prentice Hall, Harlow.

STUDY EXERCISES

19.1 Consider the value of practical work. Spend a few minutes thinking about the purpose of practical work within a specific part of your course (e.g. a particular first-year module) and then write a list of the six most important points. Compare your list with the generic list that we have provided on the website, which is based on our experience as lecturers – does it differ much from your list, which is drawn up from a student perspective?

19.2 Make a list of items required for a particular practical exercise. This exercise is likely to be most useful if you can relate it to an appropriate practical session on your course.

19.3 Check your calculator skills. Carry out the following mathematical operations, using either a hand-held calculator or a PC with appropriate 'calculator' software.

- (a) $5 \times (2 + 6)$
- (b) $(8.3 \div [6.4 - 1.9]) \times 24$ (to four significant figures)
- (c) $(1 \div 32) \times (5 \div 8)$ (to three significant figures)
- (d) $1.2 \times 10^5 + 4.0 \times 10^4$ in scientific notation (see p. 501)
- (e) $3.4 \times 10^{-2} - 2.7 \times 10^{-3}$ in 'normal' notation (i.e. conventional notation, not scientific format) and to three decimal places.

(See also the numerical exercises in Chapter 75.)

Answers to these study exercises are available at go.pearson.com/uk/he/resources

20 Working safely in the lab

Following health and safety legislation

– In the UK, the *Health & Safety at Work, etc. Act 1974* provides the main legal framework for health and safety. The *Control of Substances Hazardous to Health (COSHH) Regulations 2002* imposes specific legal requirements for risk assessment wherever hazardous chemicals or biological agents are used, with Approved Codes of Practice for the control of hazardous substances, carcinogens and biological agents, including pathogenic microbes.

Definitions

Hazard – the intrinsic ability of a substance or biological agent to cause harm.

Risk – the likelihood that a substance or biological agent might be harmful under specific circumstances.

Distinguishing between hazard and risk – one of the *hazards* associated with water is drowning. However, the *risk* of drowning in a few drops of water is negligible.

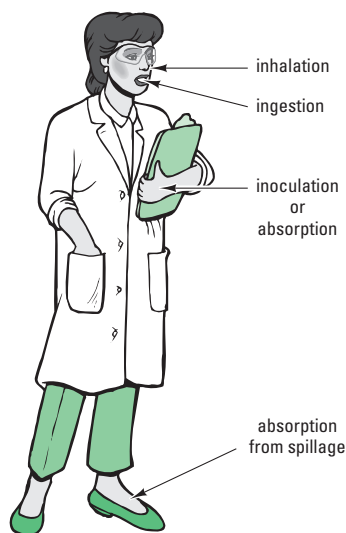


Fig. 20.1 Major routes of entry of harmful substances into the body.

You must carry out all practical work with safety in mind, to minimise the risk of harm to yourself and to others. Observe the instructions of staff at all times, but take responsibility yourself where necessary. *Safety is everyone's responsibility.*

KEY POINT Health and safety law requires institutions to provide a working environment that is safe and without risk to health. Where appropriate, training and information on safe working practices must be provided.

Assessing risk

The most widespread approach to safe working practice involves the use of risk assessment, which aims to establish:

- **The intrinsic chemical, biological and physical hazards**, together with any maximum exposure limits (MELs) or occupational exposure standards (OESs), where appropriate. Chemical manufacturers provide data sheets listing the hazards associated with particular chemical compounds, while pathogenic (disease-causing) microbes are categorised according to their ability to cause illness (p. 264).
- **The risks involved**, by taking into account the amount of substance to be used, the way in which it will be used and the possible routes of entry into the body (Fig. 20.1).

KEY POINT It is important to understand the difference between the intrinsic *hazards* of a particular substance and the *risks* involved in its use in a particular exercise.

- **The persons at risk**, and the ways in which they might be exposed to hazardous substances, including accidental exposure (spillage).
- **The steps required to prevent or control exposure.** Ideally, a non-hazardous or less hazardous alternative should be used. If this is not feasible, you should use adequate control measures, for example, a fume cupboard or other containment system. Personal protective equipment (for example, lab coats, safety glasses) must be used in addition to such containment measures. A safe means of disposal is also required.

The outcome of the risk-assessment process must be recorded and appropriate safety information must be passed on to those at risk. For most practical classes, risk assessments will have been carried out in advance by the person in charge: the information necessary to minimise your risks may be given in the practical schedule. Make sure you know how your department provides such information and that you have read the appropriate material before you begin your practical work. You should also pay close attention to the person in charge at the beginning of the practical session, as they may emphasise the major hazards and risks. In project work, you will need to be involved in the risk assessment process along with your supervisor, before you carry out any practical work.

Following basic rules for fieldwork

safety – If you are involved in any field activities make sure you understand your objectives, the potential hazards and appropriate responses to them before you set out.

In addition to specific risk assessments for individual practical sessions, most institutions will have a safety handbook, or equivalent, giving general details of safe working practices, together with the names and telephone numbers of safety personnel, first aiders, hospitals, etc. Make sure you read the relevant sections of this document and follow any instructions given.

Boxes 20.1 and 20.2 provide advice for representative scenarios where prompt and decisive action is required. They should help you plan how you might respond if a problem occurs when you are in the lab. However, each incident will be different in nature – while you should try to follow the general guidance, you should always react appropriately to the specific conditions you encounter, and in all situations seek and follow the instructions of trained personnel. Always make sure that you have read and understood the risk assessment documentation relevant to your activity, so that you are ready to respond to potential hazards. Any specific advice in the risk assessment supersedes the general guidance given.

Observing basic rules for laboratory work

- **Find out about safety equipment and rules.** All laboratories should display notices telling you where to find the first-aid kit and who to contact in case of accident/emergency.
- **Wear appropriate protective clothing at all times** – a clean lab coat (buttoned up), plus safety glasses if there is any risk to the eyes.
- **Make sure you know what to do in case of fire**, including exit routes, how to raise the alarm, and where to gather on leaving the building. Remember that the most important consideration at all times is human safety: do not attempt to fight a fire unless it is safe to do so (Box 20.1).
- **Never smoke, eat or drink in any laboratory**, because of the risks of contamination by inhalation or ingestion (Fig. 20.1).
- **Know the chemical hazards warning symbols** (Fig. 20.2).
- **Never use your mouth to pipette any liquid.** Use a pipette filler (see p. 143) or, if appropriate, a pipettor (p. 143).
- **Take care when handling glassware** – see p. 148 for details.
- **Use a fume hood for hazardous chemicals.** Make sure that it is working and then open the front only as far as is necessary: many fume hoods are marked with a maximum opening.
- **Always use the minimum quantity of any hazardous materials.**
- **Work in a logical, tidy manner and minimise risks by thinking ahead.**
- **Do not misuse any safety equipment.**
- **Always clear up at the end of each session.** This is an important aspect of safety, encouraging responsible laboratory work.
- **Dispose of waste in appropriate containers.** Most labs will have bins for sharps, glassware, hazardous solutions and radioactive waste.
- **Report all accidents**, even those appearing insignificant – your department will have a formal recording procedure to comply with safety legislation.

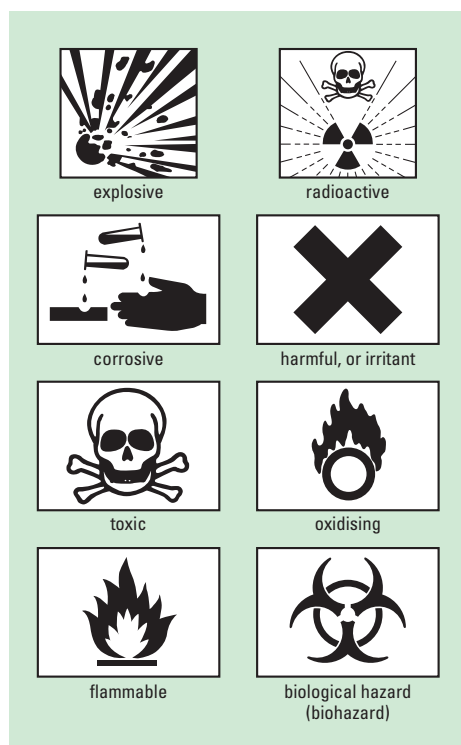


Fig. 20.2 Warning labels for specific chemical hazards. These appear on suppliers' containers and on tape used to label working vessels.

Box 20.1 How to deal with health and safety incidents in the lab

In all cases, try to stay calm. Take a few moments to assess the situation and think of the best way to react.

A. Spillages of hazardous chemicals in the lab

1. Alert your fellow students to the spillage, and where it is (they should stand clear).
2. Report the spillage immediately to a responsible person (demonstrator, technician or member of staff), providing details of the identity of the spilled material, how much was spilled and where.
3. Do not attempt to clean up the spillage unless you have been instructed to do so. The responsible person will use methods such as pH neutralisation, containment, absorption and decontamination. Any residues must be disposed of appropriately.
4. Ensure the event is reported appropriately in the appropriate incident book.

B. Contamination of a person by a chemical

1. Report the incident immediately to staff and follow their advice.
2. Try to remove the chemical and any contaminated clothing. Take care not to touch or spread the chemical.
3. If the chemical was in contact with unbroken skin, wash thoroughly with clean water. Do not rub or wipe.
4. If the person suffered a minor wound, stabilise bleeding and ask a first-aider to help. See also E below for more advice on physical injuries.
5. If the chemical was in contact with an eye, use an eye wash. Do not rub eyes.
6. If the chemical entered the person's mouth, wash it out thoroughly. Do not swallow. If the chemical has been swallowed, water should be drunk. Do not encourage vomiting.

C. Release of potentially biohazardous material

You will be unlikely to be working with extremely dangerous microorganisms (Chapter 33, p. 235), so the advice below applies mainly to low-grade biohazards. It also applies to biological toxins.

1. Where appropriate, prevent the release of further material.
2. Inform a responsible person.
3. Prevent others from entering the area.

4. Use disinfectant where appropriate – leave it to act for at least 10 minutes before wiping up.
5. Wash thoroughly and put on new personal protective equipment if appropriate, especially gloves and a face mask or respirator.
6. Dispose of used protective items as appropriate

D. Fire in a laboratory

1. Alert those around you by shouting 'FIRE!' loudly. Find the nearest (safe) fire alarm and break the glass to set off the alarm.
2. Close doors to confine the fire, then leave by the nearest exit and go to the assembly point. Do not leave this area until told it is safe to do so. When the fire service arrives, be ready to give as many details of the incident (including exact location) as you can.
3. Only try to fight a small fire using fire extinguishers or a fire blanket if it appears completely safe to do so. Fire extinguishers should be directed at the base of a fire. Always maintain a safe exit route.
4. If you hear a fire alarm, respond immediately, alerting those around you. *Never* assume an alarm is false. Avoid smoke or fumes when leaving the area.

E. Physical injury

Seek the guidance of a first-aider, or in severe cases, the local medical service. If no trained person is immediately present:

1. Assess the nature of the injury and ensure the cause of the injury is no longer a risk.
2. Call the relevant health service.
3. If the person is bleeding seriously, stem the bleeding using pressure. Apply a clean dressing to bandage the wound. A tourniquet should only be applied by a trained person in instances of life-threatening blood loss, where direct pressure is ineffective.
4. If a limb is twisted or appears to be broken, do not move the person until professional help arrives.
5. If someone is burned, cool the area with cold running water for at least 20 minutes. Seek medical help. Cover burns loosely with cling-film or a non-fluffy dressing. Do not apply any ointments or sprays.
6. If someone has fainted or is unconscious, but breathing, place them in the recovery position. If not breathing and you've had training, carry out cardiopulmonary resuscitation (CPR).

Understanding the framework for research involving gene modification – In the UK, the *Genetically Modified Organisms (Contained Use) Regulations 2014* define the legal requirements for risk assessment and for the notification of work involving genetic manipulation; The Advisory Committee on Genetic Manipulation provides guidance on the use of genetically manipulated organisms; and The Health and Safety Executive (HSE) has specific responsibility for the operation of these regulations and is the regulatory authority for genetic manipulation in the UK.

Carrying out genetic engineering and molecular genetics

Additional legal constraints apply to practical work involving genetic manipulation. A specific risk assessment must be carried out for any experiment where a cell or organism is modified by genetic engineering techniques (Chapters 66–69) involving the insertion of DNA into a cell or organism in which it does not normally occur. Before any practical work can be carried out, it must be authorised by the establishment's genetic manipulation safety committee and notified to the relevant authority. Such work must be carried out with appropriate containment, to prevent the accidental release of genetically modified organisms into the environment.

Practicals in molecular genetics will involve some of the techniques of genetic manipulation. Typically, these will be examples of 'self-cloning' where recombinant DNA molecules are constructed from fragments of DNA which naturally occur in that organism; for example, the transformation of laboratory strains of *Escherichia coli* using a pUC plasmid (p. 250).

Text references

Various sources of information were consulted when compiling Boxes 20.1 and 20.2, including:

American Chemical Society: Guide for Chemical Spill Response Planning in Laboratories Available: <https://www.acs.org/content/acs/en/about/governance/committees/chemicalsafety/publications/guide-for-chemical-spill-response.html>; *NHS Health A-Z: Acid and Chemical Burns* Available: <https://www.nhs.uk/conditions/acid-and-chemical-burns/>; *NHS inform: first aid*. Available: <https://www.nhsinform.scot/tests-and-treatments/emergencies/first-aid>; *NHS inform: first aid*. Available: <https://www.nhsinform.scot/tests-and-treatments/emergencies/first-aid>; *Infections at work* <https://www.hse.gov.uk/biosafety/infection.htm> [See section on 'Laboratories'] *University of Western Australia: Laboratory emergency response procedures* Available: <https://www.safety.uwa.edu.au/incidents-injuries-emergency/procedures/>; *US Forestry Service: If you get lost* Available: <https://www.fs.usda.gov/visit/know-before-you-go/if-you-get-lost>. (All sources last accessed 11/03/21).

Sources for further study

Anon. *Biosafety – Resources* Available: <http://www.hse.gov.uk/biosafety/information.htm> Last accessed 29/04/21. [Provides links to relevant resources.]

Anon. *COSHH basics*. Available: <https://www.hse.gov.uk/coshh/basics/index.htm> Last accessed 29/04/21. [UK Health and Safety Executive website giving an overview of the principles of risk assessment and management.]

Health and Safety Executive (2020) *Control of Substances Hazardous to Health (COSHH)*. Available: <https://www.hse.gov.uk/coshh/> Last accessed 29/04/21.

Wooley, D.P. and Byers, K.B. (2017) *Biological Safety: Principles and Practices*, 5th edn. ASM Press, Washington.

STUDY EXERCISES

20.1 Test your knowledge of safe working procedures.

After reading the appropriate sections of this book, can you remember the following:

- (a) The four main steps involved in the process of risk assessment?
- (b) The major routes of entry of harmful substances into the body?
- (c) The warning labels for the major chemical hazard symbols (either describe them or draw them from memory)?
- (d) The international symbol for a biohazard?
- (e) The international symbol for radioactivity?

20.2 Locate relevant health and safety features in a laboratory. Find each of the following in one of the laboratories used as part of your course (draw a simple location map, if this seems appropriate):

- (a) fire exit(s)
- (b) fire-fighting equipment
- (c) first-aid kit
- (d) 'sharps' container
- (e) container for disposal of broken glassware
- (f) eye wash station (where appropriate).

20.3 Investigate the health and safety procedures in operation at your university. Can you find out the following?

- (a) Your university's procedure in case of fire.
- (b) The colour coding for fire extinguishers in your department and their recommendations for use.
- (c) The accident-reporting procedure used in your department.
- (d) Your department's Code of Safe Practice relating to a specific aspect of bioscience, e.g. working with micro-organisms.

20.4 Carry out risk assessments for specific chemical hazards. Look up the hazards associated with the use of the following chemicals and list the appropriate protective measures required to minimise risk during use in a lab class:

- (a) formaldehyde solution, used as a preservative for animal tissue, to be used for microscopic examination (Chapter 25)
- (b) acetone, used as a solvent for the quantitative analysis of plant pigments (Chapter 64)
- (c) sodium hydroxide, used in solid form to prepare a dilute solution to be used for pH adjustment (Chapter 24).

Answers to these study exercises are available at go.pearson.com/uk/he/resources

21 Basic laboratory skills

Finding information on using equipment – this chapter details a range of basic instruments that are used to support common laboratory procedures. Later chapters dealing with specific methods of analysis cover the underlying theory and basic operational procedures for relevant equipment. However, you should note that it is only possible to give general explanations – manufacturer's specific instructions should always be followed.

Examples The following are examples of lab equipment covered in this book:

- **Balance** – This chapter
- **Centrifuge** – Chapter 44
- **Chromatographic systems** – Chapter 48
- **Colorimeter** – Chapter 46
- **Electrophoresis equipment** – Chapters 50 & 51
- **Fluorometer** – Chapter 47
- **Freeze dryer** – This chapter
- **Gas chromatograph** – Chapter 48
- **Haemocytometer** – Chapter 34
- **Homogeniser** – Chapter 42
- **Infrared gas analyser** – Chapter 64
- **Laminar flow cabinet** – Chapter 33
- **Light meter** – Chapter 45
- **Light microscope** – Chapters 25 and 26
- **Mass spectrometer** – Chapter 47
- **NMR spectrometer** – Chapter 47
- **Osmometer** – Chapter 23
- **Oxygen electrode** – Chapter 52
- **pH meter** – Chapter 24
- **Pipettor** – Chapter 22
- **Scintillation counter** – Chapter 54
- **Spectrophotometer** – Chapter 46
- **Stereoscopic (dissecting) microscope** – Chapter 26
- **Thermal cycler (for PCR analysis)** – Chapter 67
- **Vernier calipers** – This chapter

As a student working in a practical class or carrying out a project in a research lab, you will be expected to learn a wide range of techniques, including the operation of analytical instruments and the use of potentially hazardous chemicals. It is essential that you follow the protocol for each method exactly, for the following reasons:

- **for safe working practice**, so you do not endanger yourself or others
- **for successful results**, as these often depend on the fine detail of procedures
- **for reproducibility of data**, because standardisation of methods leads to lower rates of error.

KEY POINT You will generally receive detailed instructions the first time you use a technique or instrument, but thereafter you will probably be expected to carry out the same procedures on your own. If unsure, however, *always* ask for guidance.

Using laboratory equipment

Specialised apparatus is used in most practical classes and in project work. For practicals, the instruments available may be simplified versions of research equipment, often with fewer options for adjustment and constructed to withstand use by inexperienced operators. Examples include 'student' versions of microscopes (Chapter 26), centrifuges (Chapter 44) and spectrometers (Chapter 46). When carrying out project work in a research lab, it is likely that you will have access to equipment of greater sophistication (and value). Do not assume that this works in exactly the same way as other equipment you may have used; although the principles of operation may be similar, effective use of each instrument will require that you follow the instructions exactly as described. Optimise your technique by gaining a basic understanding of how each instrument is designed to operate, from the underpinning theory, to the specifics of its use with different sample types. You should treat all equipment with respect, as insensitive use of controls can result in errors in results, or worse, damage.

Handling chemicals

These are widely used in biomolecular sciences, for example, as reagents, stains and inhibitors or as components of growth media or diagnostic tests. Dispensing and weighing out chemicals (Box 21.1) and making up solutions (Chapter 23) are important skills required for many biomolecular investigations. Because many chemicals are corrosive, poisonous or carcinogenic, your first duty when working with them is to work with safety in mind (Chapter 20). If you need to find out about the properties of chemicals, the Merck Index (Royal Society of Chemistry, 2020) and the CRC Handbook of Chemistry and Physics (Rumble, 2020) are useful sources of information on the physical and biological properties of chemicals, including melting and boiling points, solubility and toxicity (Fig. 21.1). Detailed advice on how to work with chemicals is provided in Box 21.1.

Box 21.1 How to work with laboratory chemicals**A. Selection**

Chemicals are supplied in various degrees of purity and this is always stated on the manufacturer's containers. Suppliers differ in the names given to the grades and there is no conformity in purity standards. Very pure chemicals cost more – sometimes a lot more – and should only be used if the situation demands. If you need to order a chemical, your department will have a defined procedure for doing this.

B. Safe working with chemicals

In practical classes, the person in charge has a responsibility to inform you of any hazards associated with the use of chemicals. For routine practical procedures, a risk assessment (p. 125) will have been carried out by a member of staff and relevant safety information will be included in the practical schedule: an example is shown in Table 21.1.

In project work, your first duty when using an unfamiliar chemical is to find out about its properties, especially those relating to safety. Your department must provide the relevant information to allow you to do this. If your supervisor has filled out the risk assessment form, read it carefully before signing.

You should always treat chemicals as potentially dangerous, following these general precautions:

- **Do not use any chemical until you have considered the risks involved** – for lab classes, you should carefully read all hazard and risk information provided before you start work. In project work, you may need to be involved in the risk-assessment process with your supervisor.
- **Wear a laboratory coat at all times** – the coat should be fully fastened and cleaned appropriately, should any chemical compound be spilled on it. Closed-toe footwear will protect your feet should spillages occur.
- **Make sure you know where the safety apparatus is kept before you begin working** – this includes eye bath, fire extinguisher, blanket, first-aid kit.
- **Wear safety glasses and gloves when working with toxic, irritant or corrosive chemicals**, and for any substances where the hazards are not yet fully characterised – make sure you understand the hazard warning signs (p. 128) along with any specific hazard-coding system used in your department. Carry out procedures with solid material in a fume hood.
- **Use aids such as pipette fillers to minimise the risk of contact with hazardous solutions** – these aids are further detailed on pp. 141–142.

- **Never smoke, eat, drink or chew gum in a lab where chemicals are handled** – this will minimise the risk of ingestion.
- **Label all solutions appropriately** – use the appropriate hazard warning information (see p. 128).
- **Report all spillages of chemicals/solutions** – make sure that spillages are cleaned up properly.
- **Store hazardous chemicals only in the appropriate locations** – for example, a spark-proof fridge is required for flammable liquids; acids and solvents should not be stored together.
- **Dispose of chemicals in the correct manner** – if unsure, ask a member of staff (do not assume that it is safe to use the lab waste bin or the sink for disposal).
- **Wash hands after any direct contact with chemicals or biochemical material** – always wash your hands at the end of a lab session.

C. Storing chemicals and solutions

Labile chemicals may be stored in a fridge or freezer. Take special care when using chemicals that have been stored at low temperature: the container and its contents must be warmed up to room temperature before use, otherwise water vapour will condense on the chemical. This may render any weighing you do meaningless and it could ruin the chemical. Other chemicals may need to be kept in a desiccator, especially if they are deliquescent.

D. Labelling

Label all stored chemicals and solutions clearly, giving the following information: the chemical name (if a solution, state solute(s), concentration(s) and pH if measured), plus any relevant hazard-warning information, the date made up and your name.

E. Spillages and disposal of chemicals

You must always clean up any spillages of chemicals, as you are the only person who knows the risks from the spilled material. If you are unsure of what to do, ask a technician or other responsible member of staff. Used or excess chemicals, including solutions, should be disposed of using appropriate procedures – ask if you are unsure. Never, simply discard them down a sink – you could cause environmental harm.

8599. Sodium Chloride. [7647-14-5] Salt; common salt. ClNa; mol wt 58.44. Cl 60.67%, Na 39.34%. NaCl. The article of commerce is also known as **table salt**, **rock salt** or **sea salt**. Occurs in nature as the mineral **halite**. Produced by mining (rock salt), by evaporation of brine from underground salt deposits and from sea water by solar evaporation: *Faith, Keyes & Clark's Industrial Chemicals*, F. A. Lowenheim, M. K. Moran, Eds. (Wiley-Interscience, New York, 4th ed., 1975) pp. 722-730. Toxicity studies: E. M. Boyd, M. N. Shanas, *Arch. Int. Pharmacodyn.* **144**, 86 (1963). Comprehensive monograph: D. W. Kaufmann, *Sodium Chloride*, ACS Monograph Series no. **145** (Reinhold, New York, 1960) 743 pp.

Cubic, white crystals, granules, or powder; colorless and transparent or translucent when in large crystals. d 2.17. The salt of commerce usually contains some calcium and magnesium chlorides which absorb moisture and make it cake. mp 804° and begins to volatilize at a little above this temp. One gram dissolves in 2.8 ml water at 25°, in 2.6 ml boiling water, in 10 ml glycerol; very slightly sol in alcohol. Its soly in water is decreased by HCl. Almost insol in concd HCl. Its aq soln is neutral. pH: 6.7-7.3. d of satd aq soln at 25° is 1.202. A 23% aq soln of sodium chloride freezes at -20.5°C (5°F). LD₅₀ orally in rats: 3.75 ± 0.43 g/kg (Boyd, Shanas).

Note: **Blusalt**, a brand of sodium chloride contg trace amounts of cobalt, iodine, iron, copper, manganese, zinc is used in farm animals.

USE: Natural salt is the source of chlorine and of sodium as well as of all, or practically all, their compds, e.g., hydrochloric acid, chlorates, sodium carbonate, hydroxide, etc.; for preserving foods; manuf soap, to salt out dyes in freezing mixtures; for dyeing and printing fabrics, glazing pottery, curing hides; metallurgy of tin and other metals.

THERAP CAT: Electrolyte replenisher; emetic; topical anti-inflammatory.

THERAP CAT (VET): Essential nutrient factor. May be given orally as emetic, stomachic, laxative or to stimulate thirst (prevention of calculi). Intravenously as isotonic solution to raise blood volume, to combat dehydration. Locally as wound irrigant, rectal douche.

Fig. 21.1 Example of a typical *Merck Index* entry showing the type of information given for each chemical.

Source: from O'Neil *et al.* (2013). Reproduced with permission from *The Merck Index*, Fifteenth Edition © 2013 by Merck & Co., Inc., Whitehouse Station, NJ, USA. All rights reserved.

Using chemicals responsibly – be considerate to others: always return storeroom chemicals promptly to the correct place. Report when supplies are getting low to the person who looks after storage/ordering. If you empty an aspirator or wash bottle, fill it up from the appropriate source.

SAFETY NOTE Before you use any chemical you must find out whether safety precautions need to be taken and complete the appropriate forms confirming that you appreciate the risks involved.

Deciding on which type of balance to use – select a balance that weighs to an appropriate number of decimal places. For example, you should use a top-loading balance weighing to one decimal place for less accurate work, and a mechanical balance where extreme accuracy is required. Note that a weight of 6.4 g on such a balance may represent a true value of between 6.350 g and 6.449 g (to three decimal places).

Table 21.1 Representative risk-assessment information for a practical exercise in molecular biology, involving the isolation of DNA (see Chapter 60)

Substance	Hazards	Comments
Sodium dodecyl sulphate (SDS)	Irritant Toxic	Wear gloves
Sodium hydroxide (NaOH)	Highly corrosive Severe irritant	Wear gloves
Isopropanol	Highly flammable Irritant/corrosive Potential carcinogen	No naked flames Wear gloves
Phenol	Highly toxic Causes skin burns Potential carcinogen	Use in fume hood Wear gloves
Chloroform	Volatile and toxic Irritant/corrosive Potential carcinogen	Use in fume hood Wear gloves

Weighing samples

Many techniques require the accurate weighing of samples. Electronic balances with digital readouts (Fig. 21.2) are easy to read and their self-taring (zeroing) feature means the mass of the weighing boat or container can be subtracted automatically before weighing an object. The most common type offers accuracy down to 1 mg over the range 1 mg to 160 g, which is suitable for most biological applications.

To operate a standard self-taring balance to weigh a small mass (< 100 g):

- 1. Check that the balance is level**, using the adjustable feet to centre the bubble in the spirit level (usually at the back or side of the machine). For accurate work, make sure a draught shield is on the balance.
- 2. Place a weighing boat or an empty vessel in the middle of the balance pan** and allow the reading to stabilise. *If the object is larger than the pan, take care that no part rests on the body of the balance or the draught shield as this will invalidate the reading.* Press the 'tare' bar to bring the reading to zero.
- 3. Place the chemical or object carefully in the vessel** (powdered chemicals should be dispensed with a suitably sized clean spatula). Take care to avoid spillages.
- 4. Allow the reading to stabilise and make a note of the value.**
- 5. If you add excess chemical, take great care when removing it.** Do not return any excess chemical to the original container – dispose of it following appropriate safety measures. Switch off the balance if you need to clean any deposit accidentally left on or around the instrument.

Larger masses should be weighed on a top-loading balance to an appropriate degree of accuracy. Take care to note the limits for the balance: while most have devices to protect against overloading, you may damage the mechanism.



Fig. 21.2 Example of a self-taring electronic balance. Note the shield to help stabilise readings by reducing air flow around the machine.

Using very sensitive balances – for extremely small masses, there are mechanical and electrical balances that can weigh down to 1 μg , but these are very delicate and must be used under supervision.

Avoiding cross-contamination when weighing – never weigh anything directly on a balance's pan: you may contaminate it for other users. Use a weighing boat or a small piece of aluminium foil. Otherwise, choose a suitable vessel such as a beaker, conical flask or aluminium tray.

Understanding calibration of liquid-in-glass thermometers – some are calibrated for use in air, others require partial immersion in liquid and others total immersion; check before use.

Measuring length and area

When measuring linear dimensions, the device you need depends on the size of object and the precision demanded (Table 21.2). For many regularly shaped objects, the perimeter and area can be estimated from linear dimensions (see p. 591). The perimeter and enclosed area of an irregular shape can be measured using a planimeter, and software tools are available for mapping areas on digital images. A simpler 'low tech' method is to trace objects onto good-quality paper or to photocopy them. If the outline is then cut round, the area can be estimated by weighing the cut-out and comparing to the mass of a piece of the same paper of known area. Avoid getting moisture from the specimen on to the paper as this will affect the reading.

Measuring and controlling temperature

Using thermometers

Temperature can be critical in many biological situations and is a key factor to monitor and control. It is especially important in relation to biochemical reactions, for example when studying enzyme function (Chapter 62). The main types of thermometer you might use are:

- **Digital** – these incorporate a sensor within a probe whose electrical resistance changes with temperature. A liquid crystal display is usually incorporated and this is easy to read without error. They can include memory and 'hold' functions that are useful when recording readings.
- **Liquid-in-glass** – this type generally uses alcohol or mercury within a narrow-bore tube and provides a reading due to temperature-dependent expansion of the liquid into the partial vacuum in the tube. The maximum-minimum version is a U-shaped tube incorporating metal rods that are pushed by the liquid to indicate highs and lows. These are reset after readings using a magnet. When using these types of thermometer, ensure your eye is level with the top of the liquid before reading from the scale (p. 142).
- **Infra-red** – these detect the radiation emitted by an object, focussing this on a thermopile which, with associated electronics, provides a reading on a digital display. They have the advantage that they are non-invasive, but the corresponding disadvantage that they measure surface rather than the internal temperatures.

Table 21.2 Suitability of devices for measuring linear dimensions

Measurement device	Suitable lengths	Degree of precision
Eyepiece graticule (light microscopy)	1 μm to 10 mm	0.5 μm
Vernier calipers	1–100 mm	0.1 mm
Ruler	10 mm to 1 m	1.0 mm
Tape measure	10 mm to 30 m	1.0 mm
Optical surveying devices	1 m to 100 m	0.1 m

SAFETY NOTE If a mercury thermometer is broken, always report the spillage immediately, as mercury is a poison. Do not touch the liquid mercury.

SAFETY NOTE There is a danger of fire whenever organic material is heated and a danger of scalding from heated liquids.

SAFETY NOTE Heating/cooling glass vessels – use heat-resistant versions (e.g. Pyrex®) where possible and always take care if heating or cooling glass vessels rapidly as they may break when heat-stressed. Freezing aqueous solutions in thin-walled glass vessels is risky because ice expansion may break the glass.

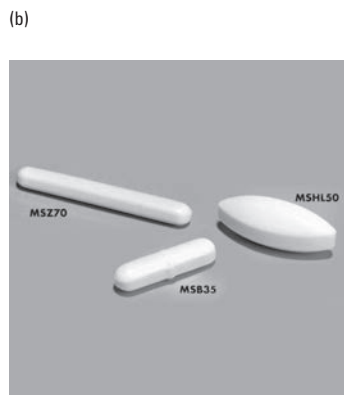


Fig. 21.3 Example of (a) a standard heater-stirrer and (b) types of magnetic stirring rods ('fleas') used with these machines (sizes range from 6 to 35 mm long). The stirrer effectively has only three controls: one for switching on and off, one for stirring speed and another for the heater thermostat.

Heating specimens

Because of the risks inherent in heating specimens, safety glasses should always be worn. Use a thermostatically controlled electric stirrer-heater if possible (Fig 21.3). If using a Bunsen burner, keep the flame well away from yourself and your clothing (tie back long hair). Use a non-flammable mat beneath a Bunsen to protect the bench. Switch off when no longer required. To light a Bunsen, close the air hole first, then apply a lit match or lighter to the top of the barrel. Open the air hole if you need a hotter, more concentrated flame: the hottest part of the flame is just above the apex of the blue cone in its centre (Fig. 33.2).

Ovens and drying cabinets may be used to dry samples or glassware. They are normally thermostatically controlled. If drying organic material for dry weight measurement, do so at about 80 °C to avoid caramelising the sample. Always state the actual temperature used as this affects results. Check that all water has been driven off by weighing until a constant mass is reached.

Cooling specimens

Fridges and freezers are used for storing stock solutions and chemicals that would either break down or become contaminated by microbes at room temperature. Typical fridge and freezer temperatures are about 4 °C and –15 °C, respectively. Ice baths can be used when reactants must be kept close to 0 °C. Most bioscience departments will have a machine that provides flaked ice for use in these baths. If common salt is mixed with ice, temperatures below 0 °C can be achieved. A mixture of ethanol and solid CO₂ will provide a temperature of –72 °C if required. To freeze a specimen quickly, immerse in liquid N₂ (–196 °C) using tongs and wearing goggles, an apron and thick gloves, as splashes will damage your eyes and skin. Always work in a well-ventilated room.

Maintaining cultures or samples at constant temperature

Thermostatically controlled temperature rooms and incubators can be used to maintain temperature at a desired level. Always check with a thermometer or thermograph (p. 413) that the thermostat is accurate enough for your study. To achieve a controlled temperature on a smaller scale, for example, with an oxygen electrode (p. 519), use a water bath. These usually incorporate heating elements, a circulating mechanism and a thermostat. Baths for sub-ambient temperatures have a cooling element.

Managing atmospheric conditions

Controlling gas composition

For accurate control of gas concentrations, use cylinders of pure gas; the contents can be mixed to give specified concentrations by controlling individual flow rates. The cylinder-head regulator (Fig. 21.4) allows you to control the pressure (and hence flow rate) of gas; adjust using the controls on the regulator or with spanners of appropriate size. Before use, ensure the regulator outlet tap is off (turn anticlockwise), then switch on at the cylinder (turn clockwise) – the cylinder dial will give you the pressure reading for the cylinder contents. Now switch on at the regulator outlet (turn clockwise) and adjust to desired pressure/flow setting. To switch off, carry out the above directions in reverse order.

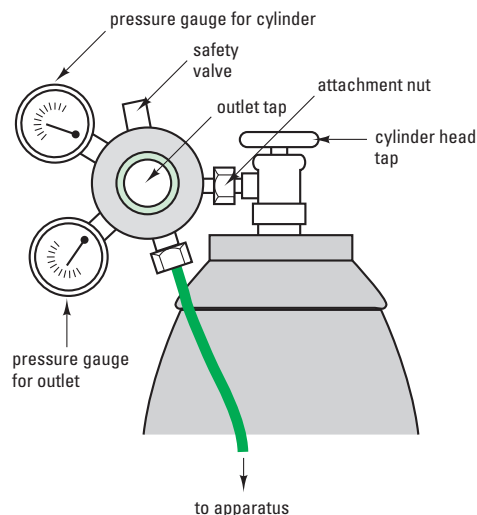


Fig. 21.4 Parts of a cylinder-head regulator. The regulator is normally attached by tightening the attachment nut clockwise; the exception is with cylinders of hydrogen, where the special regulator is tightened *anticlockwise* to avoid the chance of this potentially explosive gas being incorrectly used.

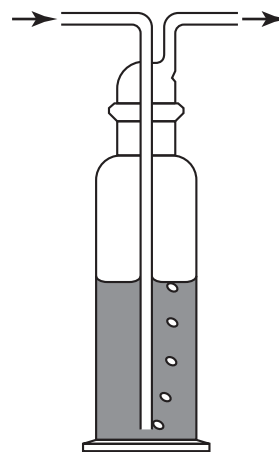


Fig. 21.5 A Dreschel bottle. The gas to be 'scrubbed' is passed through a suitable solution (e.g. aqueous KOH to remove CO_2). Several bottles may be used in series for more effective removal. The design with a 'fritted' glass exit point within the bottle gives smaller gas bubbles and is more efficient.

The atmosphere may be 'scrubbed' of certain gases by passing through a U-tube or Dreschel bottle (Fig 21.5) containing an appropriate chemical or solution.

Cleaning magnetic fleas – the flea is a potential source of cross-contamination when used to stir different solutions, so should always be cleaned carefully both before and after use.

Examples Water vapour can be removed by passing gas over dehydrated CaCO_3 , while CO_2 may be removed by bubbling through KOH solution.

Using a timer – always set the alarm *before* the critical time, so that you have adequate time to react.

Controlling pressure

Many forms of pump are used to pressurise or provide a partial vacuum, usually to force gas or liquid movement. Each has specific instructions for use. Many laboratories are supplied with 'vacuum' (suction) and pressurised air lines that are useful for procedures such as vacuum-assisted filtration or for fresh air supply (for example, as the inlet gas in Fig 21.5). Make sure you switch off the taps after use. Take special care with glass items kept at very low or high pressures – these should be contained within a metal cage to minimise the risk of injury.

Measuring time

Many experiments and observations need to be carefully timed. Most smartphones include a stopwatch function, but for safety reasons these are not generally allowed in lab environments. Large-faced stopclocks allow you to set and follow 'experimental time' and remove the potential difficulties in calculating this from 'real time' on a watch or clock. Digital versions avoid potential operator errors due to parallax effects when interpreting the position of timer hands. Many timers incorporate an alarm that you can set to warn when readings or operations must be carried out; 24-h timers are available for controlling light and temperature regimes.



Fig. 21.6 Example of a vortex mixer. The test tube is gently engaged with the orbitally vibrating rubber holder to impart a spinning motion to the liquid within. In some models, the holder can be replaced by an insert for operation with multiple sample vials, e.g. Eppendorf tubes.

SAFETY NOTE

When using a vortex mixer with open and capped test tubes – do not create too vigorous a vortex or liquid will spill from the top of the tube, creating a contamination risk.



Fig. 21.7 Mortar (bowl) and pestle (grinding tool).

Mixing and separating materials

Mixing solutions and suspensions

Various devices may be used, including:

- **Magnetic stirrers and fleas.** These are generally used to help dissolve powdered chemicals or mix solutions. Stirrers may have integral heaters (for example, Fig. 21.3(a)), while magnetic fleas come in a range of shapes and sizes (Fig. 21.3(b)) and should be selected to match the volume of the solution to be mixed. Note that stirrer speed may increase as the instrument warms up.
- **Orbital shakers and shaking water baths.** Use these to provide controlled mixing at a particular temperature, for example, for long-term incubation and cell-growth studies (p. 328).
- **Vortex mixers.** These incorporate an orbitally vibrating cup (Fig. 21.6) and are used for vigorous mixing of small volumes of solution, for example, when preparing a dilution series in test tubes (Chapter 23). Take care when adjusting the mixing speed – if the setting is too low, the test tube will vibrate rather than creating a vortex, giving inadequate mixing. If the setting is too high, the test tube may slip from your hand, or the solution may splash from the tube.
- **Bottle rollers.** For cell-culture work (Chapter 34), ensuring gentle, continuous mixing.

Homogenising

This involves breaking up and mixing specimens to give a uniform preparation and is discussed in detail in Chapter 42. Blenders are used to homogenise animal and plant material and work best when an adequate volume of liquid is present: buffer solution (pp. 166–168) may be added to specimens for this purpose. Use in short bursts to avoid overheating the motor and the sample. A mortar and pestle (Fig. 21.7) are used for grinding up specimens. Acid-washed sand grains can be added to help break up the tissues. For quantitative work with brittle samples, care must be taken not to lose material when the sample breaks into fragments.

Separation of components of mixtures and solutions

Particulate solids (for example, soils) can be separated on the basis of size using sieves. These are available in stacking forms that fit on automatic shakers. Sieves with the largest pores are placed at the top and the assembly is shaken for a fixed time until the sample separates. Suspensions of solids in liquids may be separated out by centrifugation (see p. 454) or filtration. Various forms of filter paper are available having different porosities and purities. Vacuum-assisted filtration speeds up the process and is best carried out with a filter funnel attached to a filter flask. Filtration through pre-sterilised membranes with very small pores (for example, the Millipore type, Fig. 21.8) is an excellent method of sterilising small volumes of solution. Partitioning between solvent phases is a standard preliminary method for separating components of extracts (for example, p. 441); this may be done using a separating flask (Fig. 21.9). Solvents can be removed by heating, using rotary film evaporation under low pressure and, for water, by freeze drying (Box 21.2). The last two are especially useful for heat-labile solutes.



Fig. 21.8 Using a sterile filter. In this example, the Millipore® filter is attached to a plastic syringe, used to take up and expel the liquid.

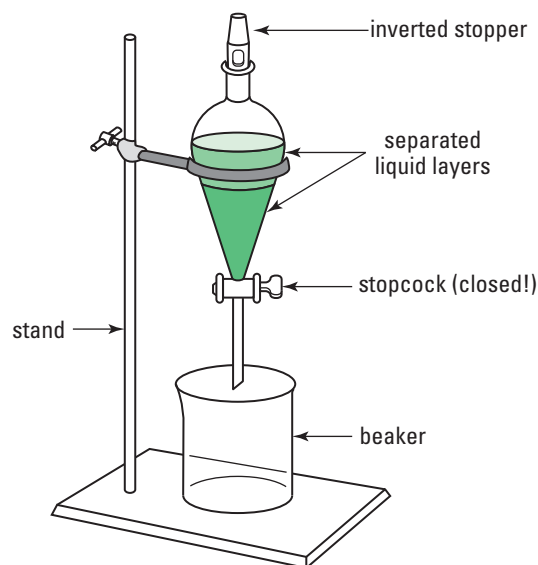


Fig. 21.9 Using a separating flask to isolate components in biphasic solutions. The following should be done in a spark-free fume cupboard: (1) introduce the sample (e.g. an aqueous tissue extract) and a second immiscible phase (e.g. an organic solvent) to the flask using a funnel, ensuring the stopcock is closed and placing a beaker beneath the outlet in case of leakage; (2) while carefully mixing the phases, invert the flask, stopper in, releasing any internal pressure from time to time using the stopcock; (3) place the flask on a stand as shown and allow the phases to separate; invert the stopper, then open the tap and collect the relevant phase(s).

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Box 21.2 How to use a freeze-dryer

In freeze drying (also known as lyophilisation or cryodesiccation), the ice in a frozen specimen is allowed to evaporate from the solid state into a partial vacuum by the process of sublimation (moving from solid to gaseous phase without liquifying). A typical freeze dryer (Fig. 21.10) consists of a chamber for drying, a vacuum system, a refrigerator-condenser system and controls for operation. To freeze-dry a specimen, you need to carry out the following procedures, some which may be automated:

- 1. Freeze the specimen**, where necessary after narcotisation and 'arrangement' (p. 246). If speed is desirable (for example, to prevent continued metabolic reactions or autolysis), you can do this by dipping the specimen in liquid nitrogen (p. 135).
- 2. Introduce the specimen into the chamber.** Care is required, as thin specimens may be extremely fragile in their frozen state; however, transfer to the chamber should be done quickly, to avoid melting of the specimen.
- 3. Seal the chamber and activate the vacuum pump.** This causes sublimation to occur – temperature reduction due to the latent heat of evaporation keeps the specimen

frozen. The temperature should be monitored and controlled to ensure the specimen is always frozen. The condenser is kept at a lower temperature than the specimen and the evaporated water is collected on this in the form of ice. This 'primary drying' phase generally removes up to 95% of the water originally present.

- 4. Raise the chamber temperature**, typically to 30–50 °C, to force 'secondary drying', which removes water chemically bound to compounds in the specimen. When this stage is complete, about 0.5% of the water originally present may remain, but the specimens will be suitable for storage.
- 5. Recover the dried specimen and store appropriately.** The partial vacuum is released, and the chamber opened for retrieval. As freeze-dried specimens can gain water from humidity in the atmosphere, you should store them carefully, as described below.

The instrument cycle described above is usually monitored via sensors and controlled by the equipment's operating system. It can take from hours to days to reach stage 5.



Fig. 21.10 Example of a bench-top freeze dryer, the BioLAB BFBT-101-B. Larger models allow bulk processing and the treatment of bigger specimens. Photo courtesy of Biolab Scientific Ltd.

STUDY EXERCISES

21.1 Consult the Merck Index (p. 132) for a chemical you may be using in a current lab exercise. Determine the limit of solubility of the chemical in water, its melting point and any other points of interest, such as its use in industry or medicine (as applicable).

21.2 Investigate the principles of operation of a laboratory instrument. Choose a piece of equipment that will be used in an upcoming practical. Research how the instrument works, what its limitations are, any safety considerations and what factors might interfere with its accuracy and precision.

21.3 Choose suitable instruments/methods. Select an appropriate technique to measure the following:

- (a) the diameter of a plant stem, to the nearest 0.1 mm
- (b) the surface temperature of a live mammal
- (c) the area of a leaf
- (d) the dimensions of a red blood cell
- (e) the time taken for a visible colour change to occur when a reagent is added to a sample
- (f) the weight of a live insect.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

22 Measuring and dispensing liquids

SAFETY NOTE Take care when using chemically hazardous liquids and solutions (flammable, corrosive, toxic, radioactive etc.) – make sure the liquid is contained properly and deal with any spillages in the correct manner (pp. 133, 322, 544).

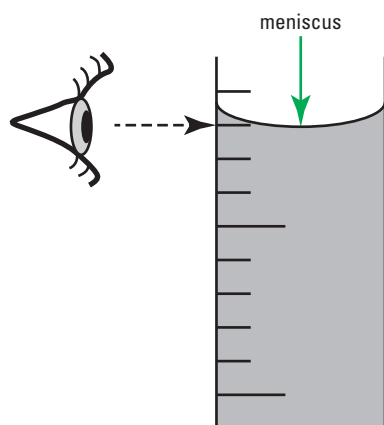


Fig. 22.1 How to read a volumetric scale (aqueous solution).

Being able to select and use equipment to deliver a particular volume of liquid is a skill that is relevant to most laboratory procedures. The apparatus you choose to measure out liquids will depend on the volume(s) being dispensed, the accuracy required and the number of times the job must be done (Table 22.1).

Certain liquids require particular attention:

- **High-viscosity liquids** are slow/difficult to dispense: allow time for all the liquid to transfer to the new vessel before reading the meniscus (Fig. 22.1).
- **Organic solvents** may evaporate rapidly, making measurements inaccurate: work quickly; seal containers without delay.
- **Solutions prone to frothing** (for example, protein and detergent solutions) can be difficult to measure and dispense: avoid forming bubbles due to over-agitation; do not transfer quickly.
- **Suspensions** (for example, cell cultures) may sediment: thoroughly mix them before transfer.

Dispensing liquids

The following types of equipment are used:

Measuring cylinders and volumetric flasks

These must be used on a level surface so that the scale is horizontal; you should first fill with solution until just below the desired mark (see Fig 22.1); then fill slowly until the meniscus is level with the mark. Allow time for any solution to run down the walls of the vessel.

Table 22.1 Criteria for choosing a method for measuring out a liquid

Method	Best volume range	Accuracy	Usefulness for repetitive measurement
Pasteur pipette	0.03–2 mL	Low	Convenient
Conical flask/beaker	25–5000 mL	Very low	Convenient
Measuring cylinder	5–2000 mL	Medium	Convenient
Volumetric flask	5–2000 mL	High	Convenient
Burette	1–100 mL	High	Convenient
Glass pipette	1–100 mL	High	Convenient
Mechanical pipettor	5–1000 μ L	High*	Convenient
Syringe	0.5–20 μ L	Medium**	Convenient
Microsyringe	0.5–50 μ L	High	Convenient
Weighing	Any (depends on accuracy of balance)	Very high	Inconvenient

* If correctly calibrated and used properly (see p. 144).

** Accuracy depends on width of barrel: large volumes are less accurate.

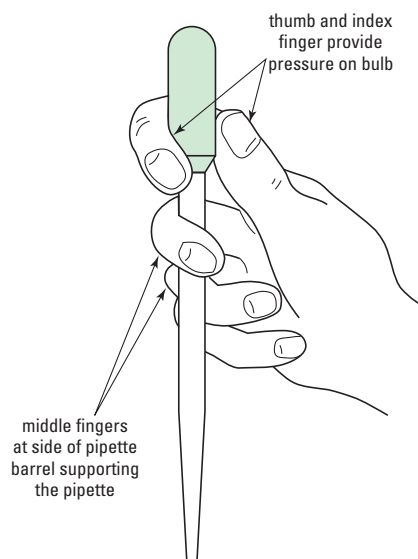


Fig. 22.2 How to hold a Pasteur pipette (Pastette).

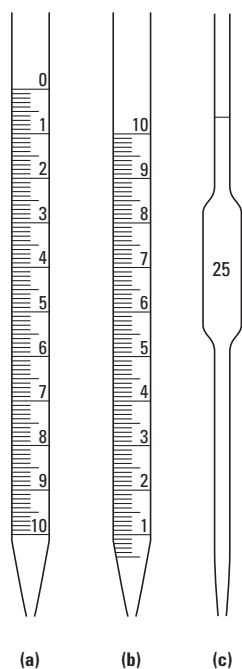


Fig. 22.3 Glass pipettes: (a) graduated pipette, reading from zero to shoulder; (b) graduated pipette, reading from maximum to tip, by gravity; (c) bulb (volumetric pipette, showing volume (calibration mark to tip, by gravity) above the bulb.

Pasteur pipettes ('Pastettes')

All-in-one plastic disposable Pastettes are preferred for most purposes, as they are less likely to break than glass Pasteur pipettes. They are available pre-sterilised, for microbiology and cell culture (Chapter 34). Hold correctly during use (Fig. 22.2) – keep the pipette vertical, with the middle fingers gripping the barrel while the thumb and index finger provide controlled pressure on the bulb. For hazardous solutions, remove the tip from the solution before fully releasing pressure on the bulb – the air taken up then helps prevent spillage. Squeeze gently to dispense individual drops. If using a glass Pasteur pipette, take care not to draw up solution into the bulb or to lie the pipette on its side, or you may cross-contaminate your solution.

Burettes

These should be mounted vertically on clamp stands – do not over-tighten. First ensure the tap is closed and fill the body with solution using a funnel. Open the tap and allow some liquid to fill the tubing below the tap before first use. Take a meniscus reading (Fig. 22.1), noting the value in your notebook. Dispense the solution via the tap and measure the new meniscus reading. The volume dispensed is the difference between the two readings. Titrations using a burette are usually performed on a magnetic stirrer (p. 138, 155).

Pipettes

These come in various designs, including graduated and bulb (volumetric) pipettes (Fig. 22.3). Take care to check the volume scale before use: some empty from full volume to zero, others from zero to full volume; some scales refer to the shoulder of the tip, others to the tip either by gravity or after blowing out.

KEY POINT For safety reasons, *never* mouth-pipette – various aids are available such as the Pi-pump, shown in Box 22.2.

Pipettors (autopipettors or micropipettes)

These come in two basic types:

- **Air displacement pipettors.** For routine work with dilute aqueous solutions. One of the most widely used examples is the Gilson Pipetman (Fig. 22.4). Box 22.1 gives practical guidance on its use.
- **Positive displacement pipettors.** For non-standard applications, including dispensing viscous, dense or volatile liquids, or certain procedures in molecular genetics, for example, the polymerase chain reaction (PCR) (p. 497), where an air displacement pipettor might create aerosols, leading to errors.

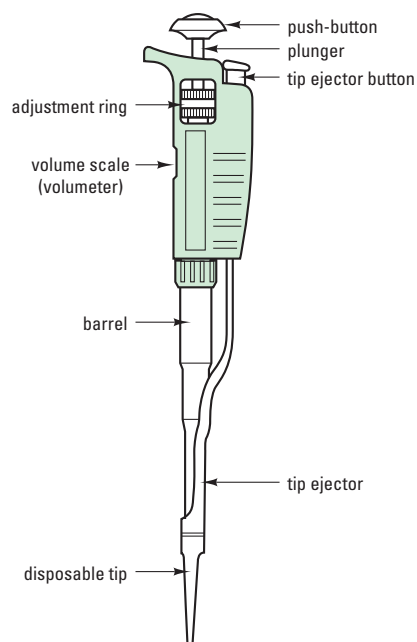


Fig. 22.4 A pipettor (autopipettor) – the Gilson Pipetman. The pipettor is designed to fit comfortably into your ‘dominant’ hand, with all four fingers wrapped around the upper (coloured) section and your thumb on the push-button.

Air displacement and positive displacement pipettors may be:

- **fixed volume** – capable of delivering a single factory-set volume
- **adjustable** – where the volume is determined by the operator across a particular range of values
- **preset** – movable between a limited number of values
- **multi-channel** – able to deliver several replicate volumes at the same time.

Whichever type you use, you must ensure that you understand the operating principles of the volume scale and the method for changing the volume delivered – some pipettors are easily misread.

A pipettor must be fitted with the correct disposable tip before use: each manufacturer produces different tips to fit particular models. Specialised tips are available for particular applications, for example, PCR (p. 497).

Syringes

Syringes should be used by placing the tip of the needle in the solution and drawing the plunger up slowly to the required point on the scale. Check the barrel to make sure no air bubbles have been drawn up. Expel slowly and touch the syringe on the edge of the vessel to remove any liquid adhering to the end of the needle. Microsyringes should always be cleaned before and after use by repeatedly drawing up and expelling pure solvent. The dead space in the syringe needle can occupy up to 4% of the nominal syringe volume. A way of avoiding such problems is to fill the dead space with an inert substance (for example, silicone oil) after sampling. Alternatively, use a syringe where the plunger occupies the needle space (small volumes only).

Balances

These can be used to weigh accurately (p. 134) how much liquid you have dispensed, based on its density. Convert mass to volume using the equation:

$$\text{mass/density} = \text{volume} \quad [22.1]$$

Densities of common solvents can be found in Rumble (2020). You will also need to know the liquid’s temperature, as density is temperature-dependent.

Holding and storing liquids

Test tubes

These are used for colour tests, small-scale reactions, preparing a dilution series (p. 146), holding cultures, etc. The tube can be sterilised by heating (p. 321, 323) and maintained in this state with a cap or cotton-wool plug.

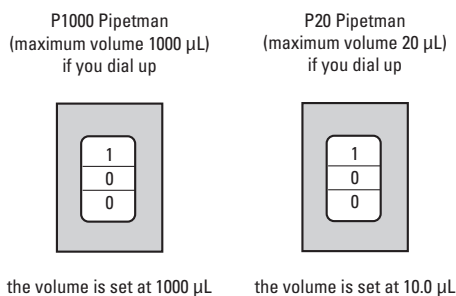
Beakers

These are used for general purposes, for example, heating a solvent while the solute dissolves, carrying out a titration.

Box 22.1 How to use a pipettor (autopipettor) to deliver accurate, reproducible volumes of liquid

A pipettor can be used to dispense volumes with accuracy and precision, by following this stepwise procedure:

- 1. Select a pipettor that operates over the appropriate range.** Most adjustable pipettors are accurate only over a particular working range and should not be used to deliver volumes below the manufacturer's specifications (minimum volume is usually 10–20% of maximum value). Do not attempt to set the volume above the maximum limit, or the pipettor may be damaged.
- 2. Set the volume to be delivered.** In some pipettors, you 'dial up' the required volume. Types like the Gilson Pipetman have a system where the scale (or 'volumeter') consists of three numbers, read from top to bottom of the barrel, and adjusted using the black knurled adjustment ring (Fig. 22.4). This number gives the first three digits of the volume scale and thus can only be understood by establishing the maximum volume of the Pipetman, as shown on the push-button on the end of the plunger (Fig. 22.4). The following examples illustrate the principle for two common sizes of Pipetman:



- 3. Fit a new disposable tip to the end of the barrel.** Make sure that it is the appropriate type for your pipettor and that it is correctly fitted. Press the tip on firmly using a slight twisting motion – if not, you will take up less than the set volume and liquid will drip from the tip during use. Tips are often supplied in boxes, for ease of use: if sterility is important, make sure you use appropriate sterile technique at all times (p. 323). *Never, ever use a pipettor without its disposable tip.*
- 4. Check your delivery.** Confirm that the pipettor delivers the correct volume by dispensing volumes of distilled water and weighing on a balance, assuming $1 \text{ mg} = 1 \mu\text{L} = 1 \text{ mm}^3$. The value should be within 1% of the selected volume. For small volumes, measure several

'squirts' together, e.g. 20 'squirts' of $5 \mu\text{L} = 100 \text{ mg}$. If the pipettor is inaccurate (p. 143) giving a biased result (e.g. delivering significantly more or less than the volume set), you can make a temporary correction by adjusting the volumeter scale down or up accordingly (the volume *delivered* is more important than the value *displayed* on the volumeter), or have the pipettor recalibrated. If the pipettor is imprecise (p. 143), delivering a variable amount of liquid each time, it may need to be serviced. After calibration, fit a clean (sterile) tip if necessary.

- 5. Draw up the appropriate volume.** Holding the pipettor *vertically*, press down on the plunger/push-button until a resistance (spring-loaded stop) is met. Then place the end of the tip in the liquid. Keeping your thumb on the plunger/push-button, release the pressure slowly and evenly: watch the liquid being drawn up into the tip, to confirm that no air bubbles are present. Wait a second or so, to confirm that the liquid has been taken up, then withdraw the end of the tip from the liquid. Inexperienced users often have problems caused by drawing up the liquid too quickly/carelessly. If you accidentally draw liquid into the barrel, seek assistance from your demonstrator or supervisor as the barrel will need to be cleaned before further use.
- 6. Make a quick visual check on the liquid in the tip.** Does the volume seem reasonable? (For example, a $100 \mu\text{L}$ volume should occupy approximately half the volume of a P200 tip.) The liquid will remain in the tip, without dripping, as long as the tip is fitted correctly and the pipettor is not tilted too far from a vertical position.
- 7. Deliver the liquid.** Place the end of the tip against the wall of the vessel at a slight angle ($10\text{--}15^\circ$ from vertical) and press the plunger/push-button slowly and smoothly to the first (spring-loaded) stop. Wait a second or two, to allow any residual liquid to run down the inside of the tip, then press again to the final stop, dispensing any remaining liquid. Remove from the vessel with the plunger/push-button still depressed.
- 8. Eject the tip.** Press the tip ejector button if present (Fig. 22.4). If the tip is contaminated, eject directly into an appropriate container, e.g. a beaker of disinfectant, for microbiological work, or a labelled container for hazardous solutions (p. 128). For repeat delivery, fit a new tip if necessary and begin again at step 5 above. Always make sure that the tip is ejected before putting a pipettor on the bench.

Using pipettors and syringes to deliver small volumes – if you find that your hand shakes when delivering small amounts of liquid, stabilise the main body of the pipettor/syringe with your second hand (Fig. 22.5), making sure that you do not touch the tip/needle.

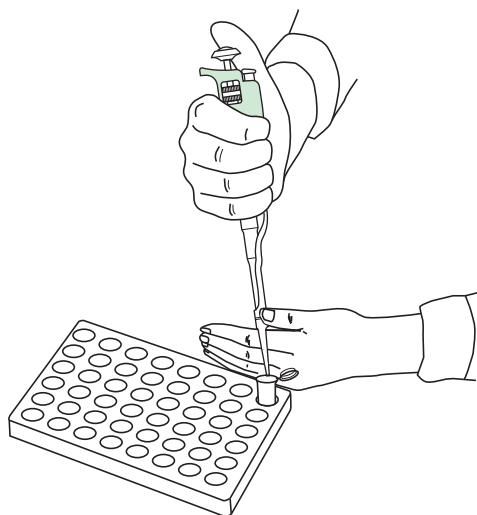


Fig. 22.5 Stabilising a pipettor (autopipettor) with the thumb of your second hand (note: thumb is positioned well away from the disposable tip).

Example

Using eqn [22.1], 9 g of a liquid with a density of $1.2 \text{ g mL}^{-1} = 7.5 \text{ mL}$.

Working with beakers and flasks –

remember that volume graduations, where present, are often inaccurate and should be used only where approximations will suffice.

Storing light-sensitive chemicals – use a coloured vessel or wrap aluminium foil around a clear vessel.

Storing an aqueous solution containing organic constituents – unless this has been sterilised or is toxic, microbes will start growing, so store for short periods in a refrigerator; older solutions may not give reliable results.

Conical (Erlenmeyer) flasks

These are used for storage of solutions: their wide base makes them stable, while their small mouth reduces evaporation and is easily sealed.

Bottles and vials

These are used when the solution needs to be sealed for safety, sterility or to prevent evaporation or oxidation. They usually have a screw top or ground-glass stopper, to prevent evaporation and contamination. Many types are available, including ‘bijou’, ‘McCartney’, ‘universal’ and ‘Winkler’.

You should clearly label all stored solutions (see p. 128), including relevant hazard information, preferably with hazard warning tape (p. 128). Seal vessels in an appropriate way, for example, using a stopper or a sealing film such as Parafilm or Nescofilm to prevent evaporation. To avoid degradation, store your solution in a fridge, but allow it to reach room temperature before use.

Preparing stock solutions

These are valuable when making up a range of solutions containing different concentrations of a reagent or if the solutions have some common ingredients. They also save work if the same solution is used over a prolonged period (for example, nutrient solution). The stock solution is more concentrated than the final requirement and is diluted as appropriate when the final solutions are made up. The principle is best illustrated with an example (Table 22.2).

Preparing dilutions

This is a skill that is used across a broad range of disciplines.

Preparing a single dilution

You may need to dilute a concentrated solution (for example, a stock solution) to give a particular mass concentration, or molar concentration. Use the following procedure:

1. **Transfer an accurate volume of stock solution to a volumetric flask**, using appropriate equipment (Table 22.1).
2. **Make up to the calibration mark with solvent** – add the last few drops from a pipette or solvent bottle, until the meniscus is level with the calibration mark.
3. **Mix thoroughly**, either by repeated inversion (holding the stopper firmly) or by prolonged stirring, using a magnetic stirrer. Make sure you add the magnetic flea *after* the volume adjustment step.

For routine work using dilute aqueous solutions where the highest degree of accuracy is not required, it may be acceptable to substitute test tubes or conical flasks for volumetric flasks. In such cases, you would calculate the volumes of stock solution and diluent required, with the assumption that the final volume is determined by the sum of the individual volumes of stock and diluent used (for example, Table 22.2). Thus, a two-fold dilution would be prepared using 1 volume of stock solution and 1 volume of diluent. The dilution factor is obtained from the ratio of the initial concentration of the stock solution and the final concentration of the diluted solution. The dilution factor can be used to determine the volumes

Table 22.2 How to prepare stock solutions – an example. Suppose you need a set of solutions 10 mL in volume containing differing concentrations of KCl, with and without reagent Q. You decide to make up a stock of KCl at twice the maximum required concentration ($50 \text{ mmol L}^{-1} = 50 \text{ mol m}^{-3}$) and a stock of reagent Q at twice its required concentration. The table shows how you might use these stocks to make up the media you require. Note that the total volumes of stock you require can be calculated from the table (end column)

Stock solutions	Volume of stock required to make required solutions (mL)						Total volume of stock required (mL)
	No KCl plus Q	No KCl minus Q	15 mmol L ⁻¹ KCl plus Q	15 mmol L ⁻¹ KCl minus Q	25 mmol L ⁻¹ KCl plus Q	25 mmol L ⁻¹ KCl minus Q	
50 mmol L ⁻¹ KCl	0	0	3	3	5	5	16
[reagent Q] × 2	5	0	5	0	5	0	15
Water	5	10	2	7	0	5	29
Total	10	10	10	10	10	10	60

Storing stock solutions – these are best kept in darkness in a refrigerator to minimise the growth of photoautotrophic microbes.

Making a dilution – use the relationship $[C_1]V_1 = [C_2]V_2$ to determine volume or concentration (see p. 150).

Removing a magnetic flea from a volumetric flask – use a strong magnet to bring the flea to the top of the flask, to avoid contamination of your solution during removal.

Using the correct volumes for dilutions – it is important to distinguish between the volumes of the various liquids: a one-in-ten dilution is obtained using 1 volume of stock solution plus 9 volumes of diluent ($1 + 9 = 10$). Note that when this is shown as a ratio, it may represent the initial and final volumes (e.g. 1:10) or, sometimes, the volumes of stock solution and diluent (e.g. 1 : 9).

Using diluents – various liquids are used, including distilled or deionised water, salt solutions, buffers, Ringer's solution (p. 360), etc., according to the specific requirements of the procedure.

of stock and diluent required in a particular instance. For example, suppose you wanted to prepare 100 mL of a solution of NaCl at 0.2 mol L^{-1} . Using a stock solution containing 4.0 mol L^{-1} NaCl, the dilution factor is $0.2 \div 4.0 = 0.05 = 1/20$ (a twenty-fold dilution). Therefore, the amount of stock solution required is $1/20$ th of 100 mL = 5 mL and the amount of diluent needed is $19/20$ th of 100 mL = 95 mL.

Preparing a dilution series

Dilution series are used in a wide range of procedures, including the preparation of standard curves for calibration of analytical instruments (p. 432), and in microbiology and immunoassay, where a range of dilutions of a particular sample is often required (pp. 333, 506). A variety of different approaches can be used:

Linear dilution series Here, the concentrations are separated by an equal amount, for example, a series containing protein at 0, 0.2, 0.4, 0.6, 0.8, $1.0 \mu\text{g mL}^{-1}$. Such a dilution series might be used to prepare a calibration curve for spectrophotometric assay of protein concentration (Box 62.1), or an enzyme assay (p. 513). Use the relationship $[C_1]V_1 = [C_2]V_2$ (p. 150) to determine the amount of stock solution required for each member of the series, with the volume of diluent being determined by subtraction.

Logarithmic dilution series Here, the concentrations are separated by a constant proportion, often referred to as the step interval.

The most common examples are:

- **Doubling dilutions** – where each concentration is half that of the previous one (two-fold step interval, \log_2 dilution series). First, make up the most concentrated solution at twice the volume required. Measure out half of this volume into a vessel containing the same volume of diluent, mix thoroughly and repeat, for as many doubling dilutions as are required. The concentrations obtained will be $1/2$, $1/4$, $1/8$, $1/16$, etc., times the original (i.e. the dilutions will be two-, four-, eight- and sixteen-fold, etc.).
- **Decimal dilutions** – where each concentration is one-tenth that of the previous one (ten-fold step interval, \log_{10} dilution series). First, make up the most concentrated solution required, with at least a

Using a linear dilution series – this is useful when preparing a calibration curve (p. 432) for spectrophotometric analysis of a substance, based on the Beer–Lambert relationship (p. 460).

Using a logarithmic dilution series – this is useful when a broad range of concentrations is required, e.g. for titration of biologically active substances (p. 161), making a plate count of a suspension of microbes (p. 332), or when a process is logarithmically related to concentration (Chapter 43).

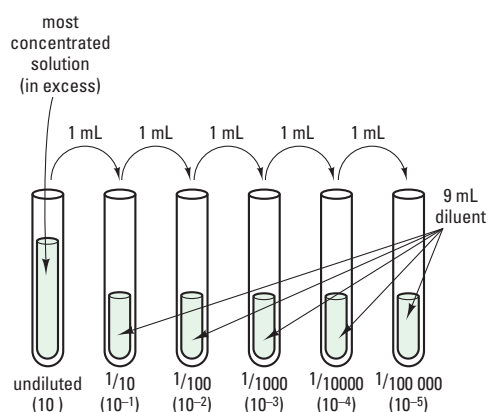


Fig. 22.6 Preparation of a dilution series. The example shown is a decimal dilution series, down to 1/100 000 (10^{-5}) of the solution in the first (left-hand) tube. Note that all solutions must be mixed thoroughly before transferring the volume to the next in the series. In microbiology and cell culture, sterile solutions and appropriate aseptic technique will be required (Chapter 33).

Creating specialised apparatus – glassware systems incorporating ground-glass connections such as Quickfit are useful for setting up combinations of standard components, e.g. for chemical reactions. In project work, you may need to adapt standard forms of glassware or source custom-made glassware.

10% excess. Measure out one-tenth of the volume required into a vessel containing nine times as much diluent, mix thoroughly and repeat.

The concentrations obtained will be 1/10, 1/100, 1/1000, etc., times the original (i.e. dilutions of 10^{-1} , 10^{-2} , 10^{-3} , etc.). To calculate the actual concentration of solute, multiply by the appropriate dilution factor.

When preparing serial doubling or decimal dilutions, it is often easiest to add the appropriate amount of diluent to several vessels beforehand, as shown in the worked example in Fig. 22.6. When preparing a dilution series, it is essential that all volumes are dispensed accurately, for example, using calibrated pipettors (p. 143), otherwise any inaccuracies will be compounded, leading to gross errors in the most dilute solutions.

Harmonic dilution series Here, the concentrations in the series take the values of the reciprocals of successive whole numbers, for example: 1, 1/2, 1/3, 1/4, 1/5, etc. The individual dilutions are simply achieved by a stepwise increase in the volume of diluent in successive vessels, for example, by adding 0, 1, 2, 3, 4 and 5 times the volume of diluent to a set of test tubes, then adding a constant unit volume of stock solution to each vessel. Although there is no dilution transfer error between individual dilutions, the main disadvantage is that the series is non-linear, with a step interval that becomes progressively smaller as the series is extended. Solutions must be thoroughly mixed before measuring out volumes for the next dilution. Use a fresh measuring vessel for each dilution to avoid contamination, or wash your vessel thoroughly between dilutions. Clearly label the vessel containing each dilution beforehand: it is easy to get confused. When deciding on the volumes required, allow for the aliquot removed when making up the next member in the series. Remember to discard any excess from the last dilution in a series if volumes are critical.

KEY POINT When preparing a dilution series using pipettes or pipettors, always use a fresh pipette or disposable tip for each dilution, to prevent carry over of solutions.

Choosing between glass and plastic vessels

Bear in mind the following points:

- **Reactivity.** Plastic vessels often distort at relatively low temperatures; they may be flammable, may dissolve in certain organic solvents and may be affected by prolonged exposure to ultraviolet (UV) light. Some plasticisers may leach from vessels and have been shown to have biological activity. Glass may adsorb ions and other molecules and then leach them into solutions, especially in alkaline conditions. Pyrex glass is stronger than ordinary soda glass and can withstand temperatures up to 500 °C.
- **Rigidity and resilience.** Plastic vessels are not recommended where volume is critical as they may distort through time: use class A volumetric glassware for accurate work, for example, preparing solutions (Chapter 23). Glass vessels are more easily broken than plastic (Box 22.2), which is particularly important for centrifugation (see p. 456).
- **Opacity.** Both glass and plastic absorb light in the UV range of the EMR spectrum (Table 22.3). Quartz should be used where this is important, for example, in cuvettes for UV spectrophotometry (see p. 461).

Table 22.3 Typical spectral cut-off values for glass and plastics (λ_{50} = wavelength at which transmission of EMR is reduced to 50%)

Material	λ_{50} (nm)
Routine glassware	340
Pyrex glass	292
Polycarbonate	396
Acrylic	342
Polyester	318
Quartz	220

SAFETY NOTE Special cleaning of glass – for an acid wash use dilute acid, e.g. 100 mmol L⁻¹ (100 mol m⁻³) HCl. Rinse thoroughly at least three times with distilled or deionised water. Glassware that must be *exceptionally* clean (e.g. for a micro-nutrient study) should be washed in a chromic acid bath, but this involves toxic and corrosive chemicals and should only be used under supervision.

- **Disposability.** Plastic items may be cheap enough to make them disposable, an advantage where there is a risk of chemical or microbial contamination.

Cleaning glass and plastic

Take care to avoid the possibility of contamination arising from prior use of chemicals or inadequate rinsing following washing. A thorough rinse with distilled or deionized water immediately before use will remove dust and other deposits and is good practice in quantitative work, but ensure that the rinsing solution is not left in the vessel. ‘Strong’ basic detergents (for example, Pyroneg) are good for solubilising acidic deposits. If there is a risk of basic deposits remaining, use an acid wash. If there is a risk of contamination from organic deposits, a rinse with Analar grade ethanol is recommended.

Glassware can be disinfected by washing with a sodium hypochlorite bleach such as Chlorox, with sodium metabisulphite or a blended commercial product such as Virkon – dilute as recommended before use and rinse thoroughly with sterile water after use. Alternatively, to sterilise, heat glassware to at least 121 °C for 15 min, in an autoclave or 160 °C for 3 h in an oven.

Box 22.2 How to work safely with glass

Many minor accidents in the laboratory are due to lack of care with glassware. You should follow these general precautions:

- **Always wear safety glasses when there is any risk of glass breakage** – e.g. when using low pressures, or heating solutions.
- **Take care when attaching tubing to glass tubes and when putting glass tubes into bungs** – always hold the tubing and glassware close together, as shown in Fig. 22.7, and wear thick gloves when appropriate.
- **Use a ‘soft’ Bunsen flame when heating glassware** – this avoids creating a hot spot, where cracks may start: always use tongs or special heat-resistant gloves when handling hot glassware (never use a rolled-up paper towel).
- **Do not use chipped or cracked glassware** – it may break under very slight strain and should be disposed of in the broken glassware bin.
- **Never carry large glass bottles/flasks by their necks** – support them with a hand underneath or, better still, carry them in a basket.
- **Do not force bungs too firmly into bottles** – these can be extremely difficult to remove. If you need a tight seal, use a screw-top bottle with a rubber or plastic seal.
- **Dispose of broken glass thoroughly and carefully** – use disposable paper towels and wear thick gloves. Always put pieces of broken glass in the correct bin.

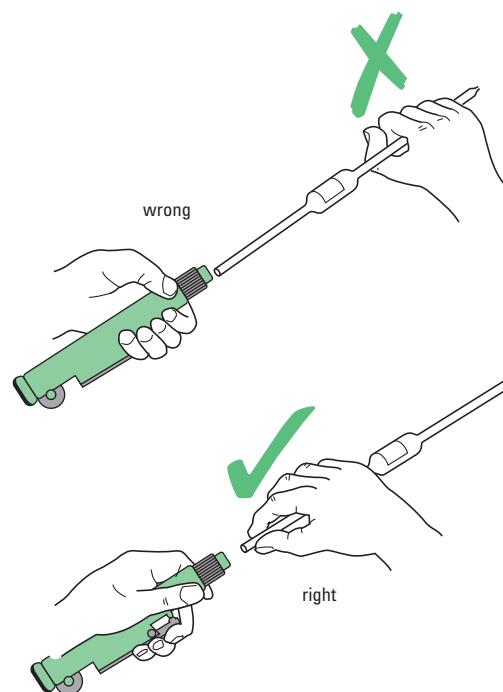


Fig. 22.7 Handling glass pipettes and tubing.

Text reference

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Sources for further study

Boyer, R.F. (2011) *Biochemistry Laboratory: Modern Theory and Techniques*, 2nd edn. Prentice Hall, New Jersey.

Hendrickson, C., Byrd, L.C., Hunter, N.W. and Henrickson, C. (2010) *A Laboratory Manual for General, Organic and Biochemistry*, 7th edn. McGraw Hill, New York.

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STUDY EXERCISES

22.1 Decide on the appropriate methods and equipment for the following procedures:

- Preparing one litre of ethanol at approximately 70% v/v in water, for use as a general preservative.
- Adding 10 μL of a sample to the well of an agarose gel during a molecular biology procedure (Chapter 67).
- Preparing a calibration standard of 100 mL of DNA, to contain 200.0 $\mu\text{g mL}^{-1}$, for spectrophotometry (Chapter 46).
- Carrying out a titration curve for a buffer solution (Chapter 24).

22.2 Practise the calculations involved in preparing dilutions (answer in each case to three significant figures).

- If you added 1.0 mL of an aqueous solution of NaCl at 0.4 mol L^{-1} to 9.0 mL of water, what would be the final concentration of NaCl in mmol L^{-1} ?
- If you added 25 mL of an aqueous solution of DNA at 10 $\mu\text{g mL}^{-1}$ to a 500 mL volumetric flask and made it up to the specified volume with water, what would be the final concentration of DNA, in ng mL^{-1} ?
- If you added 10 μL of an aqueous solution of sucrose at 200 mmol L^{-1} to a 250 mL volumetric flask and made it up to the specified volume with

water, what would be the final concentration of sucrose, in nmol mL^{-1} ?

- How would you prepare 250 mL of KCl at a final concentration of 20.0 mmol L^{-1} from a solution containing KCl at 0.2 mol L^{-1} ?
- How would you prepare $1 \times 10^{-3} \text{ m}^3$ of glucose at a final concentration of 50 $\mu\text{mol m}^{-3}$ from a stock solution containing glucose at 20.0 g m^{-3} (M_r of glucose = 180.16)?

22.3 Determine the accuracy and precision of a pipette.

Using the following data for three different models of pipettor, determine which pipettor is most *accurate* and which is most *precise* (check p. 173 if you are unsure of the definitions of these two terms). All three pipettors were set to deliver 1000 μL (1.000 mL), and ten repetitive measurements of the weight of the volume of water in grammes delivered were made using a three-place balance:

Model A pipettor:	0.986; 0.971; 0.993; 0.964; 0.983; 0.996; 0.977; 0.969; 0.982; 0.974
Model B pipettor:	1.013; 1.011; 1.010; 1.009; 1.011; 1.010; 1.011; 1.009; 1.011; 1.012
Model C pipettor:	0.985; 1.022; 1.051; 1.067; 0.973; 0.982; 0.894; 1.045; 1.062; 0.928

In your answer, you should support your conclusions with appropriate numerical (statistical) evidence (see Chapter 76 for appropriate measures of location and dispersion).

Answers to these study exercises are available at go.pearson.com/uk/he/resources

23 Preparing solutions – principles and practice

SAFETY NOTE Safety with solutions – many solutes and solvents used in biosciences are potentially toxic, corrosive, oxidising or flammable, and they may also be carcinogenic (Chapter 20). Furthermore, there is a risk of accident when using glassware to prepare, store and dispense solutions (Box 22.2).

Definitions

Mole (of a substance) –

Avogadro's constant = 6.022×10^{23} particles (atoms, molecules or ions).

Relative atomic mass (A_r) – the ratio of the average mass of one atom of an element to one-twelfth of the mass of an atom of ^{12}C .

Relative molecular mass (M_r) – the ratio of the average mass of one molecule of a chemical compound to one-twelfth of the mass of an atom of ^{12}C .

(See Chapter 28 for more detail.)

Solutions are widely used across the biomolecular sciences. Consequently, it is important to understand the underlying concepts, in order to be able to prepare and use solutions correctly in the laboratory and field. The fundamentals described here and in Chapter 22 are relevant across a broad range of disciplines.

A solution is a homogeneous liquid, formed by the addition of solutes (chemical compounds) to a solvent. Usually the solvent is water in biological systems, giving an aqueous solution. The behaviour of aqueous solutions is determined by the types of solutes involved and by their proportions, relative to the solvent. Since many laboratory exercises involve calculation of concentrations, for example, when preparing an experimental solution at a particular concentration – as described later in this chapter – or when expressing data in terms of solute concentration. Make sure that you understand the basic principles set out in the early parts of this chapter before you begin such exercises.

Understanding solute properties

Key properties of solutes that you need to take into account include:

- **Solubility** – this term refers to the ability of a solute to dissolve in a solvent and is defined in terms of the maximum amount of solute dissolved (a 'saturated solution'). Some solutes, such as hydrophobic biomolecules, dissolve poorly in water, while others – for example, inorganic salts – are highly soluble. Note that the solubility of a solute is different to the *rate* of dissolution, which depends on factors such as the size of solute particles, temperature and pH.
- **Electrolytic dissociation** – this occurs where individual molecules of a chemical compound dissociate in a solvent to give charged particles (ions). The resultant solution of ions makes the solution electrically conductive. Substances that display this effect are termed 'electrolytes'. For a strong electrolyte, for example, NaCl, dissociation in water is essentially complete, giving Na^+ and Cl^- ions. In contrast, a weak electrolyte, for example, acetic acid, will be only partly dissociated in water, depending upon the pH and temperature of the solution (p. 159).
- **Osmotic effects** – these are the result of solute particles lowering the effective concentration of the solvent (water). These effects are particularly relevant to biological systems, since membranes are far more permeable to water than to most solutes. Water moves across biological membranes from the solution with the higher effective water concentration to that with the lower effective water concentration (osmosis).
- **Non-ideal behaviour** – this occurs because solutions of real substances do not necessarily conform to the theoretical relationships predicted for dilute solutions of 'ideal' solutes. It is often necessary to take account of the non-ideal behaviour of real solutions, especially at high solute concentrations (for further information and data, see *Merck Index Online*, Royal Society of Chemistry, 2020, and Robinson and Stokes, 2002).

Expressing solute concentrations – you should use SI units wherever possible. However, you are likely to meet non-SI concentrations and you must be able to deal with these too, including interconversion.

Example A 1.0 molar solution of NaCl would contain 58.44 g NaCl (the relative molecular mass) per litre of solution.

Example A 1.0 molar solution can also be expressed as a 1.0 mol L⁻¹ solution.

Example A 0.5 molal solution of NaCl would contain $58.44 \times 0.5 = 29.22$ g NaCl per kg of water.

Example A solution of NaCl with a molality of 0.5 mol kg⁻¹ has an activity coefficient of 0.681 at 25 °C and a molal activity of $0.5 \times 0.681 = 0.340$ mol kg⁻¹.

Calculating concentrations

The term ‘concentration’ refers to the amount (in terms of either mass or moles) of *solute* (substance) per unit volume of either *solution* or, less frequently, *solvent*, in accordance with the relationship:

$$\text{concentration} = \frac{\text{amount}}{\text{volume}} \quad [23.1]$$

It is important to appreciate that both ‘amount’ and volume’ can be expressed in different units – you will need to familiarise yourself with the range of units used. In addition, you may need to convert between concentrations expressed in different units.

In SI units (p. 178), the molar concentration of a solute in a solution is expressed as the number of moles of *solute* per cubic metre volume of *solution* (mol m⁻³), which is convenient for most biological purposes. The molar concentration of a solute is usually symbolised by square brackets, for example, [NaCl]. Details of how to prepare a solution using SI and non-SI units are given in Box 23.2.

A number of alternative ways of expressing the amount of substance per unit volume of solution or solvent are in general use, and you may come across these terms in your practical work, or when reading the literature.

Molarity

This is the term used to denote molar concentration, [C], when expressed as moles of *solute* per litre volume of *solution* (mol L⁻¹). This non-SI term continues to find widespread usage, in part because of the familiarity of working scientists with the term, but also because laboratory glassware is calibrated in millilitres and litres, making the preparation of molar and millimolar solutions relatively straightforward. However, the symbols in common use for molar (M) and millimolar (mM) solutions are at odds with the SI system (Chapter 28) and many scientists now prefer to use mol L⁻¹ and mmol L⁻¹ respectively, to avoid confusion. Box 23.1 gives details of some useful approaches to calculations involving molarities.

Molality

This is used to express the concentration of solute relative to the *mass* of *solvent*, i.e. mol kg⁻¹. Molality is a temperature-independent means of expressing solute concentration, rarely used except when the osmotic properties of a solution are of interest (pp. 153, 157).

Activity (a)

This is a term used to describe the *effective* concentration of a solute. In very dilute solutions, solutes can be considered to behave according to ideal (thermodynamic) principles, with an effective concentration equivalent to the actual concentration. However, in concentrated solutions, the behaviour of solutes is often non-ideal, and their effective concentration (activity) will be less than the actual concentration [C]. The ratio between the effective concentration and the actual concentration is called the activity coefficient (γ) where

$$\gamma = \frac{a}{[C]} \quad [23.2]$$

Box 23.1 How to carry out calculations involving molar concentrations

A. Preparing a solution of defined molarity. For a solute of known relative molecular mass, M_r , the following relationship can be applied:

$$[C] = \frac{\text{mass of solute/relative molecular mass}}{\text{volume of solution}} \quad [23.3]$$

So, if you wanted to make up 200 mL (0.21) of an aqueous solution of NaCl (M_r 58.44) at a concentration of 500 mmol L⁻¹ (0.5 mol L⁻¹), you could calculate the amount of NaCl required by inserting these values into eqn [23.3]:

$$0.5 = \frac{\text{mass of solute}/58.44}{0.2}$$

which can be rearranged to

$$\text{mass of solute} = 0.5 \times 0.2 \times 58.44 = 5.844 \text{ g}$$

The same relationship can be used to calculate the concentration of a solution containing a known amount of a solute, e.g. if 21.1 g of NaCl were made up to a volume of 100 mL (0.1 L), this would give

$$[\text{NaCl}] = \frac{21.1/58.44}{0.1} = 3.61 \text{ mol L}^{-1}$$

B. Working out dilutions and concentrations. The following relationship is very useful if you are diluting (or concentrating) a solution:

$$[C_1]V_1 = [C_2]V_2 \quad [23.4]$$

where $[C_1]$ and $[C_2]$ are the initial and final concentrations, while V_1 and V_2 are their respective volumes: each pair must be expressed in the same units. Thus, if you wanted to dilute 200 mL of 0.5 mol L⁻¹ NaCl to give a final molarity of 0.1 mol L⁻¹, then, by substitution into eqn [23.4]:

$$0.5 \times 200 = 0.1 \times V_2$$

Thus $V_2 = 1000$ mL (in other words, you would have to add water to 200 mL of 0.5 mol L⁻¹ NaCl to give a final volume of 1000 mL to obtain a 0.1 mol L⁻¹ solution).

C. Determining the amount of substance in a particular volume of solution. A simple way of interconverting amounts and volumes of any particular solution is to divide the amount and volume by the same factor of ten.

Thus a molar solution of a substance contains 1 mol L⁻¹, which is equivalent to 1 mmol mL⁻¹ or 1 μ mol μ L⁻¹, or 1 nmol nL⁻¹, etc. You may find this technique useful when calculating the amount of substance present in a small volume of solution of known concentration, e.g. to calculate the amount of NaCl present in 50 μ L of a solution with a concentration (molarity) of 0.5 mol L⁻¹ NaCl:

(a) this is equivalent to 0.5 μ mol μ L⁻¹

(b) therefore 50 μ L will contain $50 \times 0.5 \mu\text{mol L} = 25 \mu\text{mol L}$.

Alternatively, you may prefer to convert to primary SI units, for ease of calculation (see Box 26.1).

The 'unitary method' (p. 594) is an alternative approach to these calculations.

Table 23.1 Activity coefficient of NaCl solutions as a function of molality. Data from Robinson and Stokes (2002)

Molality	Activity coefficient at 25 °C
0.1	0.778
0.5	0.681
1.0	0.657
2.0	0.668
4.0	0.783
6.0	0.986

Equation [23.2] can be used for SI units (mol m⁻³), molarity (mol L⁻¹) or molality (mol kg⁻¹). In all cases, γ is a dimensionless term, since a and $[C]$ are expressed in the same units. The activity coefficient of a solute is effectively unity in very dilute solution, decreasing as the solute concentration increases (Table 23.1). At high concentrations of certain ionic solutes, γ may increase to become greater than unity.

The particular use of the term 'water activity' is considered below (p. 155), since it is based on the mole fraction of *solvent*, rather than the effective concentration of *solute*.

KEY POINT Activity is often the correct expression for *theoretical* relationships involving solute concentration (e.g. where a property of the solution is dependent on concentration). However, for *practical* purposes with dilute solutions, it is sufficient to use the concentration of a solute rather than the activity, since the difference between the two terms is negligible at low concentration.

Example A 5% w/w sucrose solution contains 5 g sucrose and 95 g water (= 95 mL water, assuming a density of 1 g mL^{-1}) to give 100 g of solution.

Example A 5% w/v sucrose solution contains 5 g sucrose in 100 mL of solution. A 5% v/v glycerol solution would contain 5 mL glycerol in 100 mL of solution.

Note that when water is the solvent this is often not specified in the expression, e.g. a 20% v/v ethanol solution contains 20% ethanol made up to 100 mL of solution using water.

Example The concentration of a NaCl solution is stated as 3 ppm. This is equivalent to $3 \mu\text{g mL}^{-1}$ (3 mg L^{-1}). The relative molecular mass of NaCl is 58.44, so the solution has a concentration of $3 \times 10^{-6} \div 58.44 \text{ mol mL}^{-1} = 5.13 \times 10^{-8} \text{ mol mL}^{-1} = 0.0513 \mu\text{mol mL}^{-1} = 51.3 \mu\text{mol L}^{-1}$.

Examples Using eqn [23.1], 25 g of a substance dissolved in water to make 400 mL of solution would have a *mass* concentration (p. 149) of $25 \div 400 = 0.0625 \text{ g mL}^{-1}$ ($\equiv 62.5 \text{ mg mL}^{-1} \equiv 62.5 \text{ g L}^{-1}$).

Using eqn [23.1], 0.4 mol of a substance dissolved in water to make 0.5 L of solution would have a *molar* concentration of $0.4 \div 0.5 = 0.8 \text{ mol L}^{-1}$ ($\equiv 800 \text{ mmol L}^{-1}$).

Per cent composition (% w/w)

This is the solute mass (in g) per 100 g solution. The advantage of this expression is the ease with which a solution can be prepared, since it simply requires each component to be pre-weighed (for water, a volumetric measurement may be used, for example, using a measuring cylinder) and then mixed together. Similar terms are parts per thousand (‰), i.e. mg g^{-1} , and parts per million (ppm), i.e. $\mu\text{g g}^{-1}$.

Per cent concentration (% w/v and % v/v)

For solutes added in solid form, this is the number of grams of solute per 100 mL solution. This is more commonly used than per cent composition, since solutions can be accurately prepared by weighing out the required amount of solute and then making this up to a known volume using a volumetric flask. The equivalent expression for liquid solutes is % v/v.

Two important uses of mass/mass or mass/volume terms (including g L^{-1}) are for solutes whose relative molecular mass is unknown (for example, cellular proteins), or for mixtures of certain classes of substance (for example, total cytoplasmic salts). You should *never* use the ‘per cent’ term without specifying how the solution was prepared, i.e. by using the qualifier w/w, w/v or v/v. For mass concentrations, it is simpler to use mass per unit volume, for example, mg L^{-1} , $\mu\text{g mL}^{-1}$, etc.

Parts per million concentration (ppm)

This is a non-SI mass per volume (w/v) concentration term commonly used in quantitative analysis such as flame photometry, atomic absorption spectroscopy and gas chromatography, where low concentrations of solutes are analysed. The term ppm is equivalent to the expression of concentration as $\mu\text{g mL}^{-1}$ ($10^{-6} \text{ g mL}^{-1}$) and a 1.0 ppm solution of a substance will have a concentration of $1.0 \mu\text{g mL}^{-1}$ ($1.0 \times 10^{-6} \text{ g mL}^{-1}$).

Parts per billion (ppb) is an extension of this concentration term as ng mL^{-1} ($10^{-9} \text{ g mL}^{-1}$) and is commonly used to express concentrations of very dilute solutions. For example, the allowable concentration of arsenic in water is 0.05 ppm, but it is more conveniently expressed as 50 ppb.

Equivalent mass and normality

While these are outdated terms, you may come across them in some older texts. They apply to certain solutes whose reactions involve charged ions of a particular valency, for example, acids/alkalis and electrolytes. The equivalent mass of an ion is its relative mass divided by its valency. For acids and alkalis, the equivalent mass is the mass of substance that will provide 1 mol of either H^{+} or OH^{-} ions in a reaction. Normality expresses the equivalent mass of a solution per unit volume of solution. Thus a 1 normal solution (1 N) is one that contains one equivalent mass of a substance per litre of solution.

Preparing solutions

Solutions are usually prepared with respect to their *molar* concentrations (for example, mmol L^{-1} , or mol m^{-3}), or *mass* concentrations (for example, g L^{-1} , or kg m^{-3}), using eqn [23.1] (p. 150). The most important aspect of the process is for you to recognise clearly the units involved/required, and to prepare your solutions accordingly: for molar concentrations, you will need to find out the relative molecular mass (M_r) of the compound, so

Solving solubility problems – if a chemical does not dissolve after a reasonable time:

- check the limits of solubility for your compound (see *Merck Index Online*, Royal Society of Chemistry, 2020)
- check the pH of the solution – solubility often changes with pH, e.g. you may be able to dissolve the compound by making the solution more acidic or basic (Chapter 24).

Example Under ideal conditions, 1 mol of NaCl dissolved in 1 litre of water would give 1 mol of Na^+ ions and 1 mol of Cl^- ions, equivalent to a theoretical osmolality of 2 osmol L^{-1} (contrast with its molarity of 1 mol L^{-1}).

Definition

Osmoticum – a term given to solutes relating to their osmotic properties. This is mainly used in relation to turgor generation in plant cells (p. 155), but also in experimental situations to inert, non-permeable solutes like mannitol, which may be used to adjust the osmotic status of solutions (e.g. p. 361). The plural is **osmotica**.

Example A 1.0 mol kg^{-1} solution of NaCl has an osmotic coefficient of 0.936 at 25°C and an osmolality of $1.0 \times 2 \times 0.936 = 1.872 \text{ osmol kg}^{-1}$.

that you can determine the mass of substance required. Further advice on calculations involving molar concentrations is given in Box 23.1.

Box 23.2 explains the practical steps involved in making up a solution. The concentration you require is likely to be defined by the protocol you are following. The grade of chemical and supplier may also be specified for particular applications, where success may depend on using the same source and quality of chemical, for example, in enzyme work. To avoid waste, think carefully about the volume of solution you require for your work. However, it is always a good idea to make slightly more, because you may spill some or make a mistake when dispensing it. Try to choose one of the standard volumes for vessels (p. 147), as this will make measuring-out easier.

When preparing aqueous solutions, it is best to use distilled or deionised water, stirring the mixture to make sure all of the chemical is dissolved. Magnetic stirrers (p. 138) are the most convenient means of doing this: carefully drop a clean magnetic stirrer bar ('flea') in the beaker, avoiding splashing; place the beaker centrally on the stirrer plate, switch on the stirrer and gradually increase the speed of stirring. When the crystals or powder have completely dissolved, switch off and retrieve the flea with a magnet or another flea. Take care not to contaminate your solution when you do this and rinse the flea with distilled water. 'Obstinate' solutions may require gentle heating, but only do so if you know that the chemical will not be damaged at the temperature used. Use a stirrer-heater to keep the solution mixed as you heat it. Allow the solution to cool before you measure volume or pH as these are affected by temperature.

Understanding the osmotic properties of solutions

Osmosis is an important concept in biology, since it describes the movement of water through a selectively permeable membrane, such as those found in cells and organelles (p. 241). In the absence of pressure, water moves from a less concentrated solution to a more concentrated solution. Fig. 23.1 gives more details of the process at a cellular level, illustrating the role of turgor pressure in counterbalancing osmotically driven inward movement of water into a walled cell.

A number of alternative terms are used to express the osmotic properties of a solution, including:

Osmolarity

This non-SI expression is used to describe the number of moles of osmotically active solute particles per litre of solution (osmol L^{-1}). The need for such a term arises because some molecules dissociate to give more than one osmotically active particle in aqueous solution.

Osmolality

This term describes the number of moles of osmotically active solute particles ('osmotica') per unit mass of solvent (osmol kg^{-1}). For an ideal solute, the osmolality can be determined by multiplying the molality by n , the number of solute particles produced in solution (for example, for NaCl, $n = 2$). However, for real (i.e. non-ideal) solutes, a correction factor (the osmotic coefficient, ϕ) is used:

$$\text{osmolality} = \text{molality} \times n \times \phi \quad [23.5]$$

If necessary, the osmotic coefficients of a particular solute can be obtained from tables (for example, Table 23.2): non-ideal behaviour means that ϕ may have values >1 at high concentrations.

Box 23.2 How to make up an aqueous solution of known concentration from solid material

1. Find out or decide the concentration of chemical required and the degree of purity necessary.

2. Decide on the volume of solution required – use Box 23.1 to help with molar concentrations.

3. Find out the relative molecular mass of the chemical (M_r).
This is usually shown on suppliers' containers. If the chemical is hydrated, i.e. has water molecules associated with it, these must be included when calculating the mass required.

4. Work out the mass of chemical that will give the concentration desired in the volume required.

Suppose your procedure requires you to prepare 250 mL of 0.1 mol L^{-1} NaCl.

(a) Begin by expressing all volumes in the same units, either millilitres or litres (e.g. 250 mL as 0.25 L).

(b) Calculate the number of moles required from eqn [23.1]: $0.1 = \text{amount (mol)} \div 0.25$.

By rearrangement, the required number of moles is thus $0.1 \times 0.25 = 0.025 \text{ mol}$.

(c) Convert from mol to g by multiplying by the relative molecular mass (M_r for NaCl = 58.44).

(d) Therefore, you need to make up $0.025 \times 58.44 = 1.461 \text{ g}$ to a final volume of 250 mL of solution, using distilled water.

In some instances, it may be easier to work in SI units, though you must be careful when using exponential numbers (p. 592).

Suppose your protocol states that you need 100 mL of 10 mmol L^{-1} KCl.

(a) Start by converting this to $100 \times 10^{-6} \text{ m}^3$ of 10 mol m^{-3} KCl.

(b) The required number of mol is thus $(100 \times 10^{-6}) \times (10) = 10^{-3}$.

(c) Each mol of KCl weighs 72.56 g ($M_r = 72.56$).

(d) Therefore you need to make up $72.56 \times 10^{-3} \text{ g} = 72.56 \text{ mg KCl}$ to $100 \times 10^{-6} \text{ m}^3$ (100 mL) with distilled water.

See Box 23.1 for additional information/advice.

5. Weigh out the required mass of chemical to an appropriate accuracy. If the mass is too small to weigh to the desired degree of accuracy, consider the following options:

(a) Make up a greater volume of solution.

(b) Make up a stock solution (p. 144) that can be diluted at a later stage.

(c) Weigh the mass first, and calculate what volume to make the solution up to afterwards using eqn [23.1].

6. Add the chemical to a beaker or conical flask then add a little less water than the final amount required. If some of the chemical sticks to the paper, foil or weighing boat, use some of the water to wash it off.

7. Stir and, if necessary/appropriate, heat the solution to ensure all the chemical dissolves. You can see when this has happened by observing the disappearance of the crystals or powder.

8. If required, check and adjust the pH of the solution when cool (see Chapter 24).

9. Make up the solution to the desired volume. If the concentration needs to be accurate, use a volumetric flask (class A); if a high degree of accuracy is not required, use a measuring cylinder (class B).

(a) Pour the solution from the beaker into the measuring vessel using a funnel to avoid spillage.

(b) Make up the volume so that the meniscus comes up to the appropriate measurement line. For accurate work, rinse out the original vessel and use the rinse liquid to make up the volume.

10. Transfer the solution to a reagent bottle or a conical flask and label the vessel clearly with details of the substance, concentration, date prepared, your name or initials, plus any hazard warnings.

Osmotic pressure

This is based on the concept of a membrane permeable to water, but not to solute molecules (a selectively permeable membrane). For example, if a sucrose solution is placed on one side and pure water on the other, then a passive driving force will be created and water will travel across the membrane, through specific proteins, termed aquaporins, into the

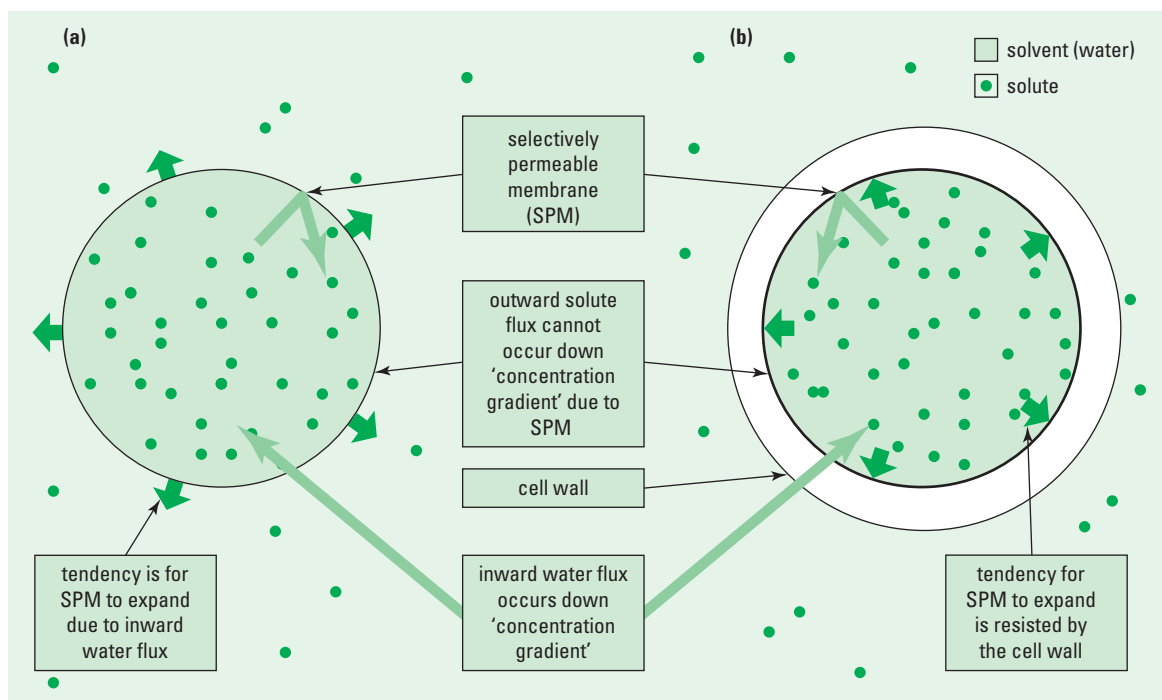


Fig. 23.1 Illustration of the forces driving solvent (water) and solute movement across a selectively permeable membrane (SPM). Energetically, both solutes and solvents tend to move down their respective 'concentration gradient' (strictly, down their chemical potential gradient). However, solute molecules cannot leave the model cells illustrated because they cannot pass through the SPM. In the situation illustrated in (a), water will tend to move from outside the cell to within because the solute molecules have effectively 'diluted' the water within the cell (illustrated by the density of point shading), creating a gradient in 'concentration' and because this molecule is able to pass through the SPM. The result will be an expansion of this model cell (short arrows). The osmotic pressure is the (theoretical) pressure that would need to be applied to prevent this expansion. If the model cell were surrounded by a cell wall, as in (b), this would resist expansion, leading to internal pressurisation (turgor pressure, p. 159).

Table 23.2 Osmotic coefficients of NaCl solutions as a function of molality. Data from Robinson and Stokes (2002)

Molality	Osmotic coefficient at 25 °C
0.1	0.932
0.5	0.921
1.0	0.936
2.0	0.983
4.0	1.116
6.0	1.271

Example A 1.0 mol kg⁻¹ solution of NaCl at 25 °C has an osmolality of 1.872 osmol kg⁻¹ and an osmotic pressure of $1.872 \times 2.479 = 4.641$ MPa.

sucrose solution, since the effective water concentration in the sucrose solution will be lower (Fig. 23.1). The tendency for water to diffuse into the sucrose solution could be counteracted by applying a hydrostatic pressure equivalent to the passive driving force. Thus, the osmotic pressure of a solution is the excess hydrostatic pressure required to prevent the net flow of water into a chamber containing the solution when separated from pure water by a selectively permeable membrane. The SI unit of (osmotic) pressure is the pascal, Pa (= kg m⁻¹ s⁻²). Older sources may use atmospheres, or bars, and conversion factors are given in Box 26.1. Osmotic pressure and osmolality can be interconverted using the expression 1 osmol kg⁻¹ = 2.479 MPa at 25 °C.

The use of osmotic pressure has been criticised as misleading, since a solution does not exhibit an 'osmotic pressure' unless it is placed on the other side of a selectively permeable membrane from pure water.

Water activity (a_w)

This term is most often used to describe the osmotic behaviour of microbial cells and foodstuffs. It is a measure of the relative proportion of water in a solution, expressed in terms of its mole fraction, i.e. the ratio of the number of moles of water (n_w) to the total number of moles of all substances (i.e. water and solutes) in solution (n_t), taking into account the molal activity coefficient of the solvent, water (i.e. γ_w):

$$a_w = \gamma_w \frac{n_w}{n_t} \quad [23.6]$$

Table 23.3 Water activity (a_w) of NaCl solutions as a function of molality. Data from Robinson and Stokes (2002)

Molality	a_w
0.1	0.997
0.5	0.984
1.0	0.967
2.0	0.932
4.0	0.852
6.0	0.760

Using an osmometer – it is vital that the sample holder and probe are clean, otherwise small amounts of the previous sample may be carried over, leading to inaccurate measurement.

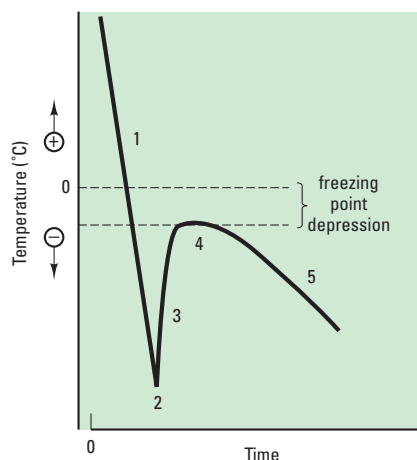


Fig. 23.2 Temperature responses of a cryoscopic osmometer. The response can be subdivided into:

1. initial supercooling
2. initiation of crystallisation
3. crystallisation/freezing
4. plateau, at the freezing point
5. slow temperature decrease.

Examples A 1.0 mol kg⁻¹ solution of NaCl at 0.1 MPa (atmospheric pressure) has a (negative) water potential of -4.641 MPa.

Pure water at 0.2 MPa pressure (about 0.1 MPa above atmospheric pressure) has a (positive) water potential of 0.1 MPa.

The water activity of pure water is unity, decreasing as solutes are added. One disadvantage of a_w is the limited change that occurs in response to a change in solute concentration: a 1.0 mol kg⁻¹ solution of NaCl has a water activity of 0.967 (Table 23.3).

Osmolality, osmotic pressure and water activity are based solely on the *osmotic* properties of a solution, with no regard for any other driving forces, for example, hydrostatic and gravitational forces. In circumstances where such other forces are important, you will need to measure a variable that takes into account these aspects of water status, namely water potential, described later in this chapter, namely, water potential, as described.

Measuring osmotic properties

Several properties vary in direct proportion to the effective number of osmotically active solute particles per unit mass of solvent; they can be used to determine the osmotic properties of a solution, typically expressed as osmolality. These properties are independent of the type of solute molecule and are termed ‘colligative properties’; they include freezing point, boiling point and vapour pressure.

An osmometer is an instrument that measures the osmolality of a solution, usually by determining the freezing point depression of the solution in relation to pure water, a technique known as cryoscopic osmometry. A small amount of sample is cooled rapidly and then brought to the freezing point (Fig. 23.2), which is measured by a temperature-sensitive thermistor probe calibrated in mosmol kg⁻¹. An alternative method is used in vapour pressure osmometry, which measures the relative decrease in the vapour pressure produced in the gas phase when a small sample of the solution is equilibrated within a chamber.

Measuring water potential (hydraulic potential)

Water potential, Ψ_w , is the most appropriate measure of osmotic status in many areas of bioscience. It is a term derived from the chemical potential of water. It expresses the difference between the chemical potential of water in the test system and that of pure water under standard conditions and has units of pressure (i.e. Pa). It is a more appropriate term than osmotic pressure because it is based on sound theoretical principles and because it can be used to predict the direction of passive movement of water, since water will flow down a gradient of chemical potential (i.e. osmosis occurs from a solution with a higher water potential to one with a lower water potential). A solution of pure water at 20 °C and at 0.1 MPa pressure (i.e. \approx atmospheric pressure) has a water potential of zero. The addition of solutes will lower the water potential (i.e. make it negative), while the application of pressure, for example, from hydrostatic or gravitational forces, will raise it (i.e. make it positive).

Often, the two principal components of water potential are referred to as the solute potential, or osmotic potential (Ψ_s , sometimes symbolised as Ψ_π or π) and the hydrostatic pressure potential (Ψ_p) respectively. For a solution at atmospheric pressure, the water potential is due solely to the presence of osmotically active solute molecules (osmotic potential) and may be calculated from the measured osmolality (osmol kg⁻¹) at 25 °C, using the relationship:

$$\Psi_w \text{ (MPa)} = \Psi_s \text{ (MPa)} = -2.479 \times \text{osmolality} \quad [23.7]$$

Measuring water potential – eqn [23.8] ignores the effects of gravitational forces – for systems where gravitational effects are important an additional term is required (Nobel, 2020).



Fig. 23.3 Vapour pressure osmometer.
Courtesy of ELITechGroup

For aquatic microbial cells, for example, algae, fungi and bacteria, equilibrated in their growth medium at atmospheric pressure, the water potential of the external medium will be equal to the cellular water potential ('isotonic') and the latter can be derived from the measured osmolality of the medium (eqn [23.7]) by osmometry (p. 155). The water potential of such cells can be subdivided into two major parts, the cell solute potential (Ψ_s) and the cell turgor pressure (Ψ_p) as follows:

$$\Psi_w = \Psi_s + \Psi_p \quad [23.8]$$

Note that Eqn [23.8] ignores the effects of gravitational forces – for systems where gravitational forces are important, an additional term is required (Nobel, 2020).

To calculate the relative contribution of the osmotic and pressure terms in eqn [23.8], an estimate of the internal osmolality is required, for example, by measuring the freezing point depression (Fig. 23.2) of expressed intracellular fluid. Once you have values for Ψ_w and Ψ_s the turgor pressure can be calculated by substitution into eqn [23.8].

For terrestrial plant cells, the water potential may be determined directly using a vapour pressure osmometer (Fig. 23.3), by placing a sample of the material within the osmometer chamber and allowing it to equilibrate. If Ψ_s of expressed sap is then measured, Ψ_p can be determined from eqn [23.8].

The van't Hoff relationship can be used to estimate Ψ_s by summation of the osmotic potentials due to the major solutes, determined from their concentrations, as:

$$\Psi_s = RTn\phi[C] \quad [23.9]$$

where RT is the product of the universal gas constant and absolute temperature (2479 J mol^{-1} at 25°C), n and ϕ are as previously defined and $[C]$ is expressed in SI terms as mol m^{-3} .

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STUDY EXERCISES

23.1 Practise calculations involving molar concentrations

What mass of substance would be required to prepare each of the following aqueous solutions (answer in grams, to three decimal places in each case):

- (a) 1 L of NaCl at a concentration of 1 molar? (M_r of NaCl = 58.44.)
- (b) 250 mL of CaCl_2 at 100 mmol L^{-1} ? (M_r of CaCl_2 = 110.99.)
- (c) 2.5 L of mannitol at 10 nmol μL^{-1} ? (M_r of mannitol = 182.17.)
- (d) 400 mL of KCl at 5% w/v?
- (e) 250 mL of glucose at 2.50 mol m^{-3} ? (M_r of glucose = 180.16.)

23.2 Practise expressing concentrations in different ways. Express all answers to three significant figures:

- (a) What is 5 g L^{-1} sucrose, expressed in terms of molarity? (M_r of sucrose = 342.3.)
- (b) What is 1.0 mol m^{-3} NaCl, expressed in g L^{-1} ? (M_r of NaCl = 58.44.)

- (c) What is 5% v/v ethanol, expressed in terms of molarity? (M_r of ethanol = 46.06 and density of ethanol at 25 °C = 0.789 gm L^{-1} .)
- (d) What is 150 mmol L^{-1} glucose, expressed in terms of per cent concentration (% w/v)? (M_r of glucose = 180.16.)
- (e) What is a 1.0 molal solution of KCl, expressed as per cent composition (% w/w)? (M_r of KCl = 74.55.)

23.3 Calculate osmolality and osmotic potentials.

- (a) Assuming NaCl, KCl and CaCl_2 behave according to ideal thermodynamic principles, what would be the predicted osmolality of a solution containing:
 - (i) NaCl alone, at 50 mmol kg^{-1} ?
 - (ii) KCl at 200 mmol kg^{-1} and CaCl_2 at 40 mmol kg^{-1} ?
 - (iii) NaCl at 100 mmol kg^{-1} , KCl at 60 mmol kg^{-1} and CaCl_2 at 75 mmol kg^{-1} ?
- (b) What is the predicted osmotic pressure and osmotic potential of each of the solutions in (a) at 25 °C? (answer to three significant figures in all cases).

Answers to these study exercises are available at go.pearson.com/uk/he/resources

24 Measuring and maintaining pH

Definitions

Acid – a compound that acts as a proton donor in aqueous solution.

Alkali – a compound that liberates hydroxyl ions when it dissociates. Since hydroxyl ions are strongly basic, this will reduce the proton concentration.

Ampholyte – a compound that can act as both an acid and a base. Water is an ampholyte since it may dissociate to give a proton and a hydroxyl ion (amphoteric behaviour).

Base – a compound that acts as a proton acceptor in aqueous solution.

Conjugate pair – an acid together with its corresponding base.

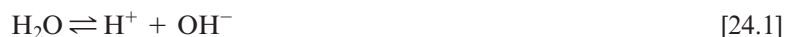
SAFETY NOTE Safe working with strong acids or alkalis – these can be highly corrosive; rinse with plenty of water, if spilled.

pH is a measure of the amount of hydrogen ions (H^+) in a solution: this affects the solubility of many substances and the activity of most biological systems, from individual molecules to whole organisms. As a result, it is important to be able to measure pH, and, especially in relation to experimental biology, to control pH via the use of appropriate buffer systems.

Taking account of background theory

It is usual to think of aqueous solutions as containing H^+ ions (protons), though protons actually exist in their hydrated form, as hydronium ions (H_3O^+). The proton concentration of an aqueous solution $[\text{H}^+]$ is affected by several factors:

- **Ionisation (dissociation) of water**, which liberates protons and hydroxyl ions in equal quantities, according to the reversible relationship:



- **Dissociation of acids**, according to the equation:



where $\text{H}-\text{A}$ represents the acid and A^- is the corresponding conjugate base. The dissociation of an acid in water will increase the amount of protons, reducing the amount of hydroxyl ions as water molecules are formed (eqn [24.1]). The addition of a base (usually, as its salt) to water will decrease the amount of H^+ , due to the formation of the conjugate acid (eqn [24.2]).

- **Dissociation of alkalis**, according to the relationship:



where $\text{X}-\text{OH}$ represents the undissociated alkali. Since the dissociation of water is reversible (eqn [24.1]), in an aqueous solution the production of hydroxyl ions will effectively act to ‘mop up’ protons, lowering the proton concentration.

Many compounds act as acids, bases or alkalis: those that are almost completely ionised in solution are usually called strong acids or bases, while weak acids or bases are only slightly ionised in solution (p. 162).

In an aqueous solution, most of the water molecules are not ionised. In fact, the extent of ionisation of pure water is constant at any given temperature and is usually expressed in terms of the ion product (or ionisation constant) of water, K_w :

$$K_w = [\text{H}^+][\text{OH}^-] \quad [24.4]$$

where $[\text{H}^+]$ and $[\text{OH}^-]$ represent the molar concentration (strictly, the activity) of protons and hydroxyl ions in solution, expressed as mol L^{-1} . At 25 °C, the ion product of pure water (Table 24.1) is $10^{-14} \text{ mol}^2 \text{ L}^{-2}$ (i.e. $10^{-8} \text{ mol}^2 \text{ m}^{-6}$). This means that the concentration of protons in solution will be $10^{-7} \text{ mol L}^{-1}$ ($10^{-4} \text{ mol m}^{-3}$), with an equivalent concentration of hydroxyl ions (eqn [24.1]). Since these values are very low and involve negative powers of 10, it is customary to use the pH scale, where:

$$\text{pH} = -\log_{10} [\text{H}^+] \quad [24.5]$$

and $[\text{H}^+]$ is the proton activity in mol L^{-1} (see p. 150).

Table 24.1 Effects of temperature on the ion product of water (K_w), H^+ ion concentration and pH at neutrality. Values calculated from Rumble (2020)

Temp. (°C)	K_w (mol ² L ⁻²)	[H ⁺] at neutrality (nmol L ⁻¹)	pH at neutrality
0	0.11×10^{-14}	33.9	7.47
4	0.17×10^{-14}	40.7	7.39
10	0.29×10^{-14}	53.7	7.27
20	0.68×10^{-14}	83.2	7.08
25	1.01×10^{-14}	100.4	7.00
30	1.47×10^{-14}	120.2	6.92
37	2.39×10^{-14}	154.9	6.81
45	4.02×10^{-14}	199.5	6.70

Example Human plasma has a typical H^+ concentration of approximately 0.4×10^{-7} mol L⁻¹ ($=10^{-7.4}$ mol L⁻¹), giving a pH of 7.4.

Table 24.2 Properties of some pH indicator dyes

Dye	Acid-base colour change	Useful pH range
Thymol blue (acid)	red–yellow	1.2–6.8
Bromophenol blue	yellow–blue	1.2–6.8
Congo red	blue–red	3.0–5.2
Bromocresol green	yellow–blue	3.8–5.4
Resazurin	orange–violet	3.8–6.5
Methyl red	red–yellow	4.3–6.1
Litmus	red–blue	4.5–8.3
Bromocresol purple	yellow–purple	5.8–6.8
Bromothymol blue	yellow–blue	6.0–7.6
Neutral red	red–yellow	6.8–8.0
Phenol red	yellow–red	6.8–8.2
Thymol blue (alkaline)	yellow–blue	8.0–9.6
Phenolphthalein	none–red	8.3–10.0

KEY POINT While pH is strictly the negative logarithm (to the base 10) of H^+ activity, in practice, H^+ concentration in mol L⁻¹ (equivalent to kmol m⁻³ in SI terminology) is most often used in place of activity, since the two are virtually the same, given the limited dissociation of H₂O. The pH scale is therefore not strictly SI: nevertheless, it continues to be of great value in biological science.

The value where an equal amount of H^+ and OH^- ions are present is termed neutrality: at 25 °C the pH of pure water at neutrality is 7.0. At this temperature, pH values below 7.0 are acidic while values above 7.0 are alkaline.

Always remember that the pH scale is a *logarithmic* one, not a *linear* one: a solution with a pH of 3.0 is not twice as acidic as a solution of pH 6.0, but one thousand times as acidic (i.e. contains 1000 times the amount of H^+ ions). Therefore, you may need to convert pH values into proton concentrations before you carry out mathematical manipulations, like averaging (see Box 76.2). For similar reasons, it is important that pH change is expressed in terms of the original and final pH values, rather than simply quoting the difference between the values: a pH change of 0.1 has little meaning unless the initial or final pH is known.

Measuring pH

pH indicator dyes

These compounds (usually weak acids) change colour in a pH-dependent manner. They may be added in small amounts to a solution, or they can be used in paper strip form. Each indicator dye usually changes colour over a restricted pH range, typically 1–2 pH units (Table 24.2): universal indicator dyes/papers make use of a combination of individual dyes to measure a wider pH range. Dyes are not suitable for accurate pH measurement as they are affected by other components of the solution including oxidising and reducing agents and salts. However, they are useful for:

- **estimating the approximate pH of a solution**
- **determining a change in pH**, for example at the end point of a titration or the production of acids during bacterial metabolism
- **establishing the approximate pH of intracellular compartments**, for example the use of neutral red as a ‘vital’ stain (p. 271).

pH electrodes

Accurate pH measurements can be made using a pH electrode, coupled to a pH meter. The pH electrode is usually a combination electrode, comprising two separate systems: an H^+ -sensitive glass electrode and a reference electrode which is unaffected by H^+ ion concentration. When this is immersed in a solution, a pH-dependent voltage between the two electrodes can be measured using a potentiometer. In most cases, the pH electrode assembly (containing the glass and reference electrodes) is connected to a separate pH meter by a cable. Box 24.1 gives details of the steps involved in making a pH measurement with a glass pH electrode and meter.

Box 24.1 How to use a glass pH electrode and meter to measure the pH of a solution

The following procedure should be used whenever you make a pH measurement: consult the manufacturer's handbook for specific information, where necessary. Do not be tempted to miss out any of the steps detailed below, particularly those relating to the effects of temperature, or your measurements are likely to be inaccurate.

- 1. Stir the test solution thoroughly before you make any measurement:** it is often best to use a magnetic stirrer. Leave the solution for sufficient time to allow equilibration at lab temperature.
- 2. Record the temperature of every solution you use,** including all calibration standards and samples, since this will affect K_w , neutrality and pH.
- 3. Set the temperature compensator on the meter to the appropriate value.** This control makes an allowance for the effect of temperature on the electrical potential difference recorded by the meter: it does *not* allow for the other temperature-dependent effects mentioned elsewhere. Basic instruments have no temperature compensator, and should only be used at a specified temperature, either 20 °C or 25 °C, otherwise they will not give an accurate measurement. More sophisticated systems have automatic temperature compensation.
- 4. Rinse the electrode assembly (Fig. 24.1) with distilled water** and gently dab off the excess water on to a clean tissue: check for visible damage or contamination of the glass electrode (consult a member of staff if the glass is broken or dirty). Also check that the solution within the glass assembly is covering the metal electrode.
- 5. Calibrate the instrument:** set the meter to 'pH' mode, if appropriate, and then place the electrode assembly in a standard solution of known pH, usually pH 7.00. This solution may be supplied as a liquid, or may be prepared by dissolving a measured amount of a calibration standard in water: calibration standards are often provided in tablet form, to be dissolved in water to give a particular volume of solution. Adjust the calibration control to give the correct reading. Remember that your calibration standards will only give the specified pH at a particular temperature, usually either 20 °C or 25 °C. If you are working at a different temperature, you must establish the actual pH of your calibration standards, either from the supplier, or from literature information.

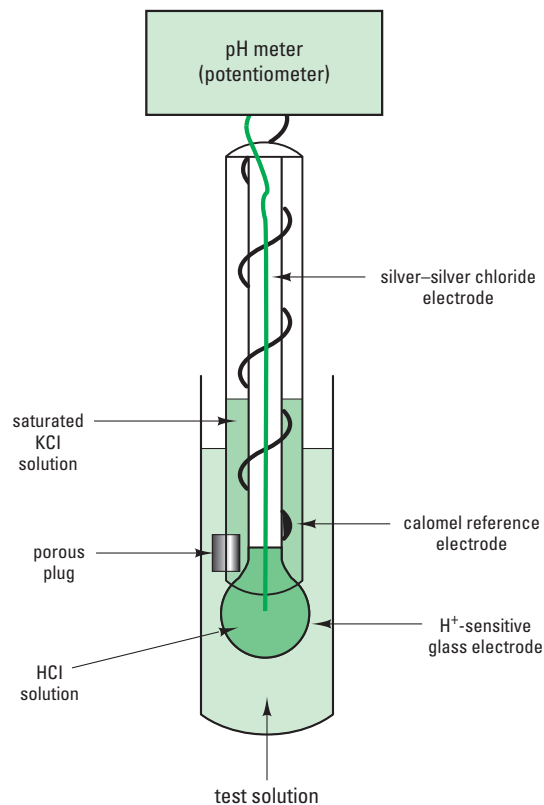


Fig. 24.1 Measurement of pH using a combination pH electrode and meter. The electrical potential difference recorded by the potentiometer is directly proportional to the pH of the test solution.

- 6. Remove the electrode assembly from the calibration solution and rinse again with distilled water:** dab off the excess water. Basic instruments have no further calibration steps (single-point calibration), while the more refined pH meters have additional calibration procedures.

If you are using a basic instrument, you should check that your apparatus is accurate over the appropriate pH range by measuring the pH of another standard whose pH is close to that expected for the test solution. If the standard does not give the expected reading, the instrument is not functioning correctly: consult a member of staff.

If you are using an instrument with a slope control function, this will allow you to correct for any deviation in electrical potential from that predicted by the theoretical

(continued)

Box 24.1 (continued)

relationship (at 25 °C, a change in pH of 1.00 unit should result in a change in electrical potential of 59.16mV) by performing a two-point calibration. Having calibrated the instrument at pH 7.00, immerse in a second standard at the same temperature as that of the first standard, usually buffered to either pH 4.00 or pH 9.00, depending upon the expected pH of your samples. Adjust the slope control until the exact value of the second standard is achieved (Fig. 24.2). A pH electrode and meter calibrated using the two-point method will give accurate readings over the pH range from 3 to 11: laboratory pH electrodes are not accurate outside this range, since the theoretical relationship between electrical potential and pH is valid.

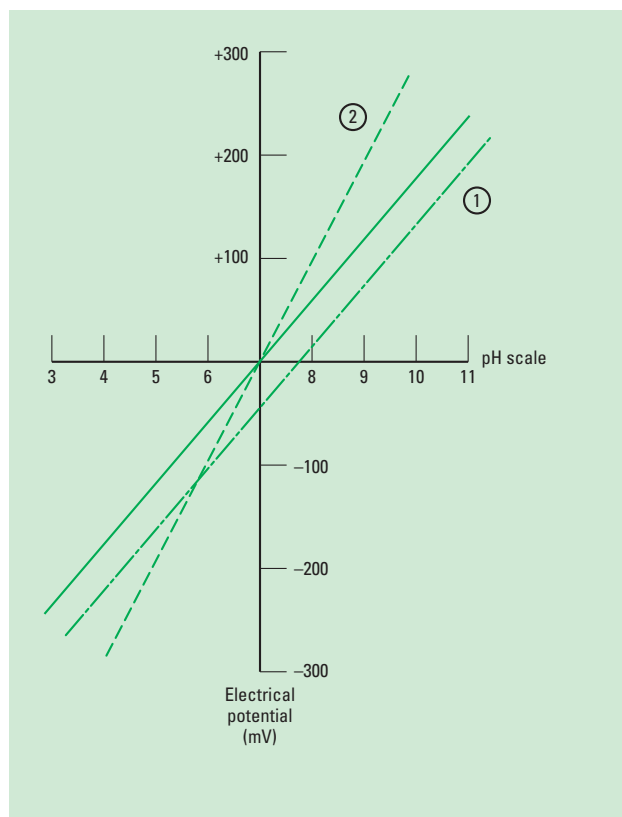


Fig. 24.2 The relationship between electrical potential and pH. The solid line shows the response of a calibrated electrode while the other plots are for instruments requiring calibration: 1 has the correct slope but incorrect isopotential point (calibration control adjustment is needed); 2 has the correct isopotential point but incorrect slope (slope control adjustment is needed).

7. Once the instrument is calibrated, measure the pH of your solution(s), making sure that the electrode assembly is rinsed thoroughly between measurements. You should be particularly aware of this requirement if your solutions contain organic biological material, e.g. soil, tissue fluids, protein solutions, etc., since these may adhere to the glass electrode and affect the calibration of your instrument. If your electrode becomes contaminated during use, check with a member of staff before cleaning: avoid touching the surface of the glass electrode with abrasive material. Allow sufficient time for the pH reading to stabilise in each solution before taking a measurement: for unbuffered solutions, this may take several minutes, so do not take inaccurate pH readings due to impatience!

8. After use, the electrode assembly must not be allowed to dry out. Most pH electrodes should be stored in a neutral solution of KCl, either by suspending the assembly in a small beaker, or by using an electrode cap filled with the appropriate solution (typically 1.0 mol L⁻¹ KCl buffered at pH 7.0). However, many labs simply use distilled water as a storage solution, leading to loss of ions from the interior of the electrode assembly. In practice, this means that pH electrodes stored in distilled water will take far longer to give a stable reading than those stored in KCl.

9. Switch the meter to zero (where appropriate), but do not turn off the power: pH meters give more stable readings if they are left on during normal working hours.

Problems (and solutions) include:

- inaccurate and/or unstable pH readings caused by cross-contamination (rinse electrode assembly with distilled water and blot dry between measurements);
- development of a protein film on the surface of the electrode (soak in 1% w/v pepsin in 0.1 mol L⁻¹ HCl for at least an hour);
- deposition of organic or inorganic contaminants on the glass bulb (use an organic solvent, such as acetone, or a solution of 0.1 mol L⁻¹ disodium ethylenediamine tetraacetic acid, respectively);
- drying out of the internal reference solutions (drain, flush and refill with fresh solution, then allow to equilibrate in 0.1 mol L⁻¹ HCl for at least an hour);
- cracks or chips to the surface of the glass bulb (use a replacement electrode).

Measuring pH – the pH of a neutral solution changes with temperature (Table 24.1), due to the enhanced dissociation of water with increasing temperature. This must be taken into account when measuring the pH of any solution and when interpreting your results.



Fig. 24.3 A pocket pH meter suitable for use in the field. Courtesy of Cole-Parmer Instrument Company Ltd.

Producing H^+ -selective glass – this key component of pH electrodes has hydronium ion selectivity due to the presence of Na_2O , CaO , SiO_2 and Al_2O_3 in the glass mix which creates chemically active binding sites in the glass.

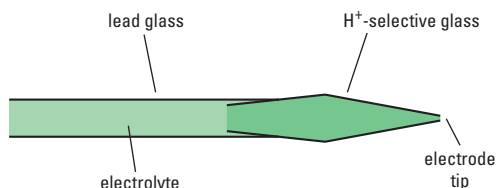


Fig. 24.4 Simplified diagram of a pH microelectrode. The H^+ -selective glass is inserted into the object of interest. The current flowing between a reference electrode in the solution bathing the object and the electrolyte within the electrode is proportional to the pH of the solution surrounding the probe.

Portable pH probes

In these devices (Fig. 24.3), sometimes called ‘pocket pH meters’, the electrodes and meter are housed within the same robust assembly, often using an H^+ -sensitive field effect transistor in place of a glass electrode, to improve durability and portability. They are easily carried, can be operated ‘one-handed’, and some models are fully waterproof and buoyant. Quoted sensitivity is ± 0.01 pH unit, but frequent recalibration is essential for continued accuracy and this is generally carried out using standard buffers at pH 4, 7 and 10. Although the sensor electrode may degrade through time, these are replaceable. The instrument is generally used by dipping the sensor in aqueous solution, but some designs of pocket pH meters operate with single drops of solution, down to 0.1 mL.

pH measurements at the cell level

Several techniques have been developed to estimate pH in intracellular compartments. At best, they can achieve μm levels of spatial resolution and sub-second time resolution. You will be unlikely to use these sensitive and specialised methods during practical classes, but you may apply them in advanced research projects or find them referred to in research papers. The range of techniques includes:

- **Microelectrodes** – typically, a H^+ -selective membrane or glass is part of a glass micropipette that is inserted into a cell as a ‘mini pH meter’ (Fig. 24.4). A tip diameter of less than $1 \mu m$ is required; so great skill is required both to construct and deploy the electrode. This approach is most appropriate for readings from large cells.
- **Weak acid or base ‘markers’** – this technique takes advantage of the fact that a lipophilic marker will penetrate a cell’s membrane-bound compartments with a concentration differential depending on the inner and outer pH (for example, p. 535). The accumulation of marker can be measured using radioactive labelling and/or fluorescence intensity, and is perhaps best suited to investigations on the function of isolated organelles.
- **Fluorescent reagents** – the ambient pH affects the wavelength spectrum of certain fluorescent compounds (for example, fluorescein) when excited by particular wavelengths of light. Accordingly, the fluorescence intensity, measured using a suitable microscope-mounted detector, can act as a proxy for cellular or organelle pH changes. The relevant compounds need to be first injected into the cell and the technique can provide real-time readings of pH changes.
- **Nuclear magnetic resonance (NMR)** – the NMR spectrum of ^{31}P changes according to pH and can provide non-destructive measurements utilising natural compounds within cells that contain this natural isotope (Chapter 47). A disadvantage is that it requires highly sophisticated and expensive equipment.

The above methods generally provide an averaged reading of pH within a particular cellular compartment. A number of techniques, such as instruments based on light addressable potentiometric sensors (LAPS), have the potential to provide ‘proton imaging’ at the sub-cellular level.

Controlling pH

Using buffers

Rather than simply measuring the pH of a solution, you may wish to *control* the pH, for example, in metabolic experiments, or in a growth medium for cell culture (p. 328). In fact, you should consider whether you need to control pH in any experiment involving a biological system, whether whole organisms, isolated cells, subcellular components or biomolecules. One of the most effective ways to control pH is to use a buffer solution.

A buffer solution is usually a mixture of a weak acid and its conjugate base. Added protons will be neutralised by the anionic base while a reduction in protons, for example, due to the addition of hydroxyl ions, will be counterbalanced by dissociation of the acid (eqn [24.2]); thus the conjugate pair acts as a ‘buffer’ to pH change. The innate resistance of most biological fluids to pH change is due to the presence of cellular constituents that act as buffers, for example, proteins, which have a large number of weakly acidic and basic groups in their amino acid side chains.

Definition

Buffer solution – one that resists a change in H^+ concentration (pH) on addition of acid or alkali.

Buffer capacity and the effects of pH

The extent of resistance to pH change is called the buffer capacity of a solution. The buffer capacity is measured experimentally at a particular pH by titration against a strong acid or alkali: the resultant curve will be strongly sigmoidal, with a plateau where the buffer capacity is greatest (Fig. 24.5). The mid-point of the plateau represents the pH where equal quantities of acid and conjugate base are present, and is given the symbol pK_a , which refers to the negative logarithm (to the base 10) of the acid dissociation constant, K_a , where

$$K_a = \frac{[H^+][A^-]}{[HA]} \quad [24.6]$$

Specifying the features of an ideal buffer

– for biological purposes a buffer would possess the following characteristics:

- impermeability to biological membranes
- biological stability and lack of interference with metabolic and biological processes
- lack of significant absorption of ultraviolet or visible light
- lack of formation of insoluble complexes with cations
- minimal effect of ionic composition or salt concentration
- limited pH change in response to temperature.

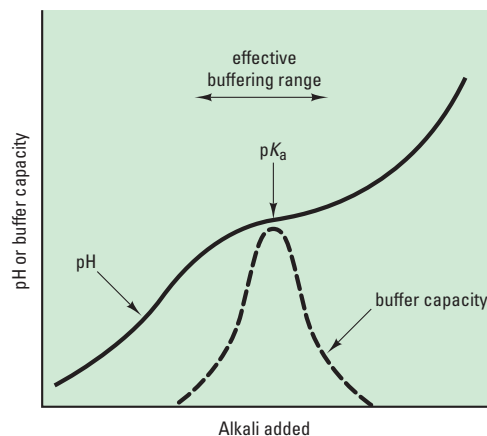


Fig. 24.5 Theoretical pH titration curve for a buffer solution. pH change is lowest and buffer capacity is greatest at the pK_a of the buffer solution.

Table 24.3 pK_a values at 25 °C and M_r of some acids and bases (upper section) and some large organic zwitterions (lower section) commonly used in buffer solutions. For polyprotic acids, where more than one proton may dissociate, the pK_a values are given for each ionisation step. Only the trivial acronyms of the larger molecules are provided: their full names can be obtained from the catalogues of most chemical suppliers

Acid or base	pK_a value(s)	M_r
Acetic acid	4.8	60.1
Boric acid	9.2	61.8
Citric acid	3.1, 4.8, 5.4	191.2
Glycylglycine	3.1, 8.2	132.1
Phosphoric acid	2.1, 7.1, 12.3	98.0
Phthalic acid	2.9, 5.5	166.1
Succinic acid	4.2, 5.6	118.1
TRIS (base)*	8.1	121.1
CAPS (free acid)	10.4	221.3
CHES (free acid)	9.3	207.3
HEPES (free acid)	7.5	238.3
MES (free acid)	6.1	213.2
MOPS (free acid)	7.2	209.3
PIPES (free acid)	6.8	302.4
TAPS (free acid)	8.4	243.3
TRICINE (free acid)	8.1	179.2

*Note that this compound is hygroscopic and should be stored in a desiccator; also see text opposite regarding its potential toxicity.

SAFETY NOTE Preparing solutions for adjusting buffer pH – you may need to make up acid and base solutions to achieve this. When making a dilute acid solution using concentrated acid, always slowly add the concentrated acid to water, not the reverse, since the strongly exothermic process can trigger a violent reaction with water. When preparing an alkali solution, typically, the alkali will be in solid form (e.g. NaOH) and addition to water will rapidly raise the temperature of the solution: use only heat-resistant glassware, cooled with water if necessary.

By rearranging eqn [24.6] and taking negative logarithms, we obtain:

$$\text{pH} = \text{p}K_a + \log_{10} \frac{[\text{A}^-]}{[\text{HA}]} \quad [24.7]$$

This relationship is known as the Henderson–Hasselbalch equation and it shows that the pH will be equal to the pK_a when the ratio of conjugate base to acid is unity, since the final term in eqn [24.7] will be zero. Consequently, pK_a is an important factor in determining buffer capacity at a particular pH. In practical terms, a buffer solution will work most effectively at pH values about one unit either side of the pK_a .

Selecting an appropriate buffer

When selecting a buffer, you should be aware of certain limitations to their use. Citric acid and phosphate buffers readily form insoluble complexes with divalent cations, while phosphate can also act as a substrate, activator or inhibitor of certain enzymes. Both of these buffers contain biologically significant quantities of cations, for example, Na^+ or K^+ . TRIS (Table 24.3) is often toxic to biological systems: due to its high lipid solubility it can penetrate membranes, uncoupling electron transport reactions in whole cells and isolated organelles. In addition, it is markedly affected by temperature, with a tenfold increase in H^+ concentration from 4 °C to 37 °C. A number of zwitterionic molecules (having both positive and negative groups) have been introduced to overcome some of the disadvantages of traditional buffers. These newer compounds are often referred to as ‘Good buffers’, to acknowledge the work of Dr N.E. Good: HEPES is one of the most useful zwitterionic buffers, with a pK_a of 7.5 at 25 °C.

These zwitterionic substances are usually added to water as the free acid: the solution must then be adjusted to the correct pH with a strong alkali, usually NaOH or KOH. Alternatively, they may be used as their sodium or potassium salts, adjusted to the correct pH with a strong acid, for example, HCl. Consequently, you may need to consider what effects such changes in ion concentration may have in a solution where zwitterions are used as buffers. In addition, zwitterionic buffers can interfere with protein determinations (for example, Lowry method, p. 439).

Figure 24.6 shows a number of traditional and zwitterionic buffers and their effective pH ranges. When selecting one of these buffers, aim for a pK_a that is in the direction of the expected pH change (Table 24.3). For example, HEPES buffer would be a better choice of buffer than PIPES for use at pH 7.2 for experimental systems where a pH increase is anticipated, while PIPES would be a better choice where acidification is expected.

Preparing buffer solutions

Having selected an appropriate buffer, you will need to make up your solution to give the desired pH. You will need to consider two factors:

1. **The ratio of acid and conjugate base** required to give the correct pH.
2. **The amount of buffering required;** buffer capacity depends upon the absolute quantities of acid and base, as well as their relative proportions.

Table 24.4 Preparation of sodium phosphate buffer solutions for use at 25 °C. Prepare separate stock solutions of (a) disodium hydrogen phosphate and (b) sodium dihydrogen phosphate, both at 200 mol m⁻³. Buffer solutions (at 100 mol m⁻³) are then prepared at the required pH by mixing together the volume of each stock solution shown in the table, then diluting to a final volume of 100 mL using distilled or deionised water

Required pH (at 25 °C)	Volume of stock (a) Na ₂ HPO ₄ (mL)	Volume of stock (b) NaH ₂ PO ₄ (mL)
6.0	6.2	43.8
6.2	9.3	40.7
6.4	13.3	36.7
6.6	18.8	31.2
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.8	4.2
8.0	47.4	2.6

Understanding buffering capacity –

remember that buffer solutions will only work effectively if they have sufficient buffering capacity to resist the change in pH expected during the course of the experiment. Thus a weak solution of HEPES (e.g. 10 mmol L⁻¹, adjusted to pH 7.0 with NaOH) will not be able to buffer the growth medium of a dense suspension of cells for more than a few minutes.

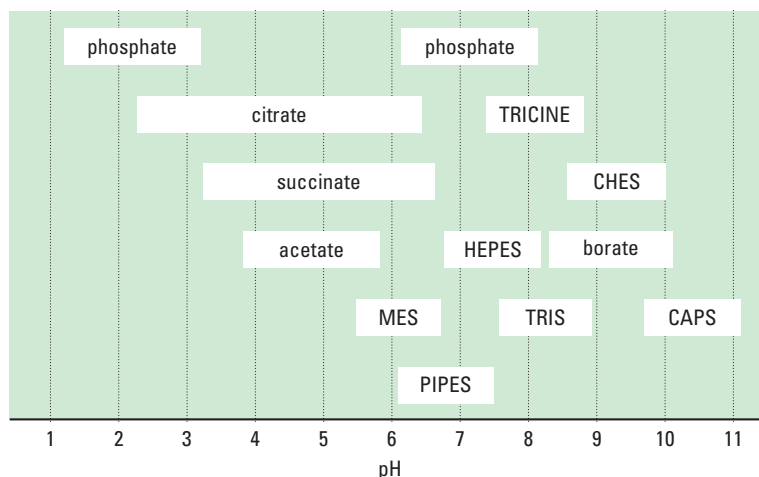


Fig. 24.6 Useful pH ranges of some commonly used buffers.

In most instances, buffer solutions are prepared to contain between 10 mmol L⁻¹ and 200 mmol L⁻¹ of the conjugate pair. Although it is possible to calculate the quantities required from first principles using the Henderson–Hasselbalch equation, there are sources that tabulate the amount of substance required to give a particular volume of solution with a specific pH value for a range of buffers (for example, Anon, 2006, 2011). For traditional buffers, it is customary to mix stock solutions of acidic and basic components in the correct proportions to give the required pH (Table 24.4). For zwitterionic acids, the usual procedure is to add the compound to water, then bring the solution to the required pH by adding a specific amount of strong alkali or acid (obtained from tables). Alternatively, the required pH can be obtained by dropwise addition of alkali or acid, using a meter to check the pH, until the correct value is reached. When preparing solutions of zwitterionic buffers, the acid may be relatively insoluble. Do not wait for it to dissolve fully before adding alkali to change the pH – the addition of alkali will help bring the acid into solution, but make sure it has all dissolved before the desired pH is reached.

Finally, when preparing a buffer solution based on tabulated information, always confirm the pH with a pH meter before use.

Text references

Anon. (2007) *pH Theory and Practice*. Available: <https://ats-scientific.com/uploads/products/docs/radiometer-analytical-ph-theory.pdf> Last accessed 30/04/21.

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Sources for further study

Anon. (2003) *Buffers. A Guide for the Preparation and use of Buffers in Biological Systems*. Available: http://wolfson.huji.ac.il/purification/PDF/Buffers/Calbiochem_Buffers_Booklet.pdf Last accessed 30/04/21.

Ruiz, F. (2020) *What is a Biological Buffer and how to Choose the Best Buffer for your Experiment*. Available: <https://www.goldbio.com/articles/article/what-is-a-biological-buffer-and-how-to-choose-the-best-buffer-for-your-experiment> Last accessed 30/04/21.

STUDY EXERCISES

24.1 Practise interconverting pH values and proton concentrations. Express all answers to three significant figures.

- What is pH 7.4 expressed as $[H^+]$ in mol L^{-1} ?
- What is pH 4.1, expressed as $[H^+]$ in mol m^{-3} ?
- What is the pH of a solution containing H^+ at $2 \times 10^{-5} \text{ mol L}^{-1}$?
- What is the pH of a solution containing H^+ at $10^{-12.5} \text{ mol L}^{-1}$?
- What is the pH of a solution containing H^+ at $2.8 \times 10^{-5} \text{ mol m}^{-3}$?

24.2 Decide on a suitable buffer to use. In the following instances, choose a buffer that would be suitable:

- Maintaining the pH at 8.5 during an enzyme assay of a cell-free extract at 25 °C.
- Keeping a stable pH of 6.5 in an experiment to measure the uptake of radiolabelled glucose by a dense suspension of *E. coli*.

- Carrying out an assay of photosynthetic activity at pH 7.2 at temperatures of 10 °C, 20 °C and 30 °C.
- Stabilising pH at 5.5 during enzyme extraction, in a solution where you intend to measure total protein concentration at a later stage.

24.3 Practise using the Henderson–Hasselbalch equation. What are the relative proportions of deprotonated (A^-) and protonated (HA) forms of each substance at the following pH values:

- acetic acid ($pK_a = 4.8$) for use in an experiment at pH 3.8?
- boric acid ($pK_a = 9.2$) for use in an experiment at pH 9.5?
- HEPES ($pK_a = 7.5$) for use in an experiment at pH 8.1?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

25 Understanding microscopy

Table 25.1 Comparison of microscope types. Resolution is that obtained by a skilled user. LM, light microscope; SEM, scanning electron microscope; TEM, transmission electron microscope

Property	Type of microscope		
	LM	TEM	SEM
Resolution	200 nm	1 nm	10nm
Depth of focus	Low	Medium	High
Field of view	Good	Limited	Good
Specimen preparation (ease)	Easy	Skilled	Easy
Specimen preparation (speed)	Rapid	Slow	Quite rapid
Relative cost of instrument	Low	High	High

Definitions

Resolution – the ability to distinguish between two points on the specimen – the better the resolution, the ‘sharper’ the image.

Contrast – the difference in intensity perceived between different parts of an image.

Many features of interest in biological systems are too small to be seen by the naked eye and can only be observed with a microscope. The three main forms of instrument are the light microscope, the transmission electron microscope (TEM) and the scanning electron microscope (SEM). These microscopes are compared in Table 25.1 and their suitability for observing cells and organelles is shown in Table 25.2.

All microscopes consist of a coordinated system of lenses arranged so that a magnified image of a specimen is seen by the viewer (Fig. 25.1). The main differences are the wavelengths of electromagnetic radiation used to produce the image, the nature and arrangement of the lens systems and the methods used to view the image. The resolution of the image is affected by the lens design and inversely related to the wavelength of radiation used. The contrast of the image can be enhanced (a) by the use of stains, and (b) by adjusting microscope settings, usually at the expense of resolution.

KEY POINT Microscopes allow objects to be viewed with increased resolution and contrast, but only if they are set up and used correctly.

Using light microscopy

Two forms of the standard light microscope, the binocular (compound) microscope and the dissecting microscope, are described in detail in Chapter 26. These are the instruments most likely to be used in routine practical work. Figure 25.2(a) shows a typical image from a light microscope.

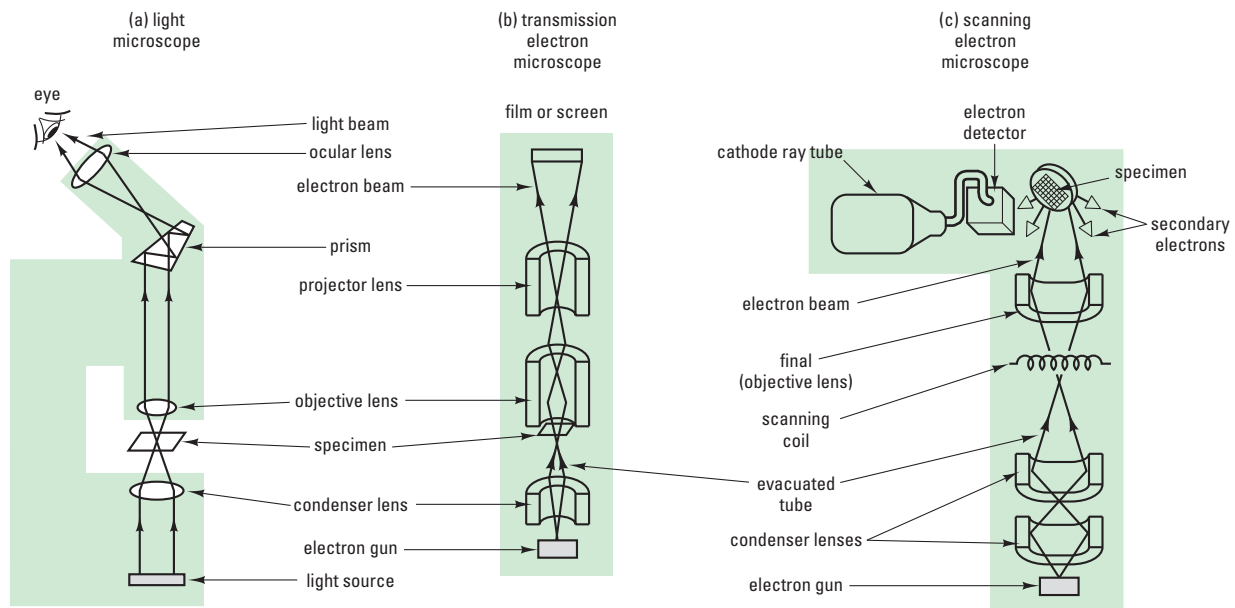


Fig. 25.1 Simplified diagrams of light and electron microscopes. Note that the electron microscopes are drawn upside-down to aid comparison with the light microscope.

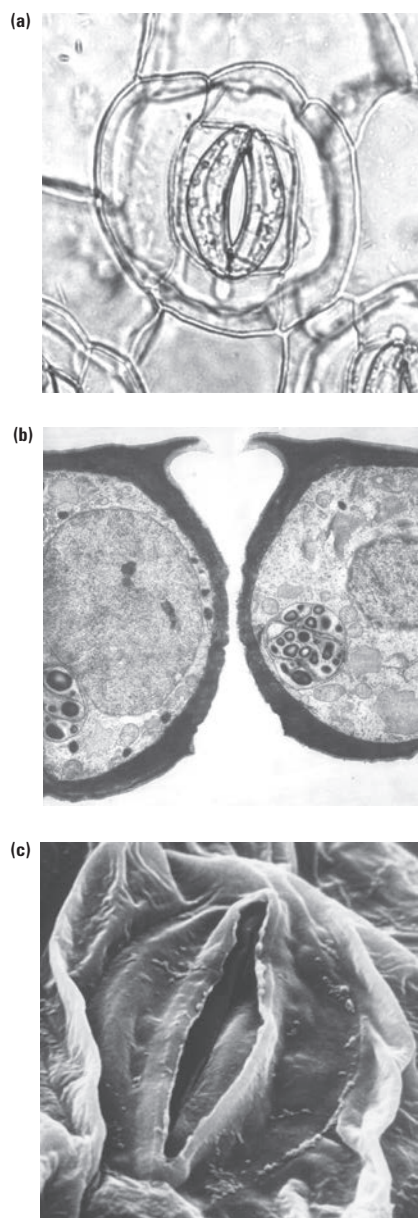


Fig. 25.2 Examples of images of a similar specimen (the stomatal complex of *Commelina communis* L.) obtained using different microscopic techniques: (a) light microscopy (surface view); (b) transmission electron microscopy (transverse section through guard cell pair at mid pore); and (c) scanning electron microscopy (surface view). As an indication of scale, the width of a single guard cell is about 10 μm .

In more advanced project work, you may use one or more of the following more sophisticated variants of light microscopy to improve image quality:

- **Dark-field illumination** involves a special condenser that causes reflected and diffracted light from the specimen to be seen against a dark background. The method is particularly useful for near-transparent specimens and for delicate structures like flagella. Care must be taken with the thickness of slides used – air bubbles and dust must be avoided and immersion oil must be used between the dark-field condenser and the underside of the slide.
- **Ultraviolet and fluorescence microscopy** use light of UV wavelengths, the former to increase resolution, and the latter to make certain fluorescent substances (fluorophores) emit light of a characteristic longer (visible) wavelength. Custom light sources, lenses and mountants are required for both forms, and filters must be used to prevent damage to users' eyes. The high selectivity of fluorescence microscopy comes in part from the rarity of the fluorescence response. Fluorophores can be intrinsic to the specimen (for example, chlorophyll or porphyrins) or applied fluorescent dyes (stains) that bind to specific cell components, revealing their location (for example fluorescein). The most common form is epifluorescence microscopy, where the excitation beam is directed through the objective lens and fluorescence is visualised using the same lens.
- **Phase-contrast microscopy** is useful for increasing contrast when viewing transparent specimens. It is superior to dark-field microscopy because a better image of the interior of specimens is obtained. Phase contrast operates by causing constructive and destructive interference effects in the image, visible as increased contrast. Adjustments must be made, using a phase telescope in place of the eyepiece, for each objective lens and a matching phase condenser, and the microscope must be set up carefully to give optimal results.
- **Nomarski or differential interference contrast (DIC) microscopy** gives an image with a three-dimensional quality. However, the relief seen is optical rather than morphological, and care should be taken in interpreting the result. One of the advantages of the technique is the extremely limited depth of focus that results: this allows 'optical sectioning' of a specimen.
- **Polarised-light microscopy** can be used to reveal the presence and orientation of optically active components within specimens (for example, starch grains, cellulose fibres), showing them brightly against a dark background.
- **Confocal microscopy** allows three-dimensional views of cells or thick sections. A finely focused laser is used to create electronic images of layered horizontal 'slices', usually after fluorescent staining. Images can be viewed individually or reconstructed to provide a 3-D computer-generated image of the whole specimen.

Typical specimen preparation procedures are summarised in Fig 25.3.

Table 25.2 Dimensions of some typical cells and organelles with an indication of suitable forms of microscopy for observing them. LM, light microscope; SEM, scanning electron microscope; TEM, transmission electron microscope. Column 2 data after Rubbi (1994)

Cell or organelle	Approximate diameter or width (μm)	Suitable form of microscopy
Prokaryote cell	0.15–5	LM, SEM, TEM
Eukaryote cell	10–100	LM, SEM, TEM
Fungal hypha	5–20	LM, SEM, TEM
Nucleus	5–25	LM, SEM, TEM
Mitochondrion	1–10	SEM, TEM
Chloroplast	2–8	LM, SEM, TEM
Golgi apparatus	1	SEM, TEM
Lysosome/ peroxisome	0.2–0.5	SEM, TEM
Plant cell wall	0.1–10	LM, SEM, TEM

Using electron microscopy

These instruments offer an image resolution up to 200 times better than light microscopes (Table 25.1, Fig. 25.2) because they utilise radiation of shorter wavelength in the form of an electron beam. The electrons are produced by a tungsten filament operating in a vacuum and are focused by electromagnets. TEM and SEM differ in the way in which the electron beam interacts with the specimen: in TEM, the beam passes through the specimen (Fig. 25.1(b)), while in SEM the beam is scanned across the specimen and is reflected from the surface (Fig. 25.1(c)). In both cases, the beam must fall on a fluorescent screen before the image can be seen. Permanent images (‘electron micrographs’) are produced after focusing the beam on photographic film (Figs 25.2(b) and (c)).

You are unlikely to use either type of electron microscope as part of undergraduate practical work because of the time required for specimen preparation (Fig. 25.3) and the need for detailed training before these complex machines can be operated correctly. However, electron microscopy is extremely important in understanding cellular and subcellular structures, and you may be shown electron micrographs with one or more of the following goals:

- to demonstrate cell ultrastructure (TEM)
- to show surface features of organisms, when cells are ‘freeze-fractured’, then coated (SEM)
- to investigate changes in the number, size, shape and condition of cells and organelles (TEM)
- to carry out quantitative studies of cell and organelle disposition (TEM).

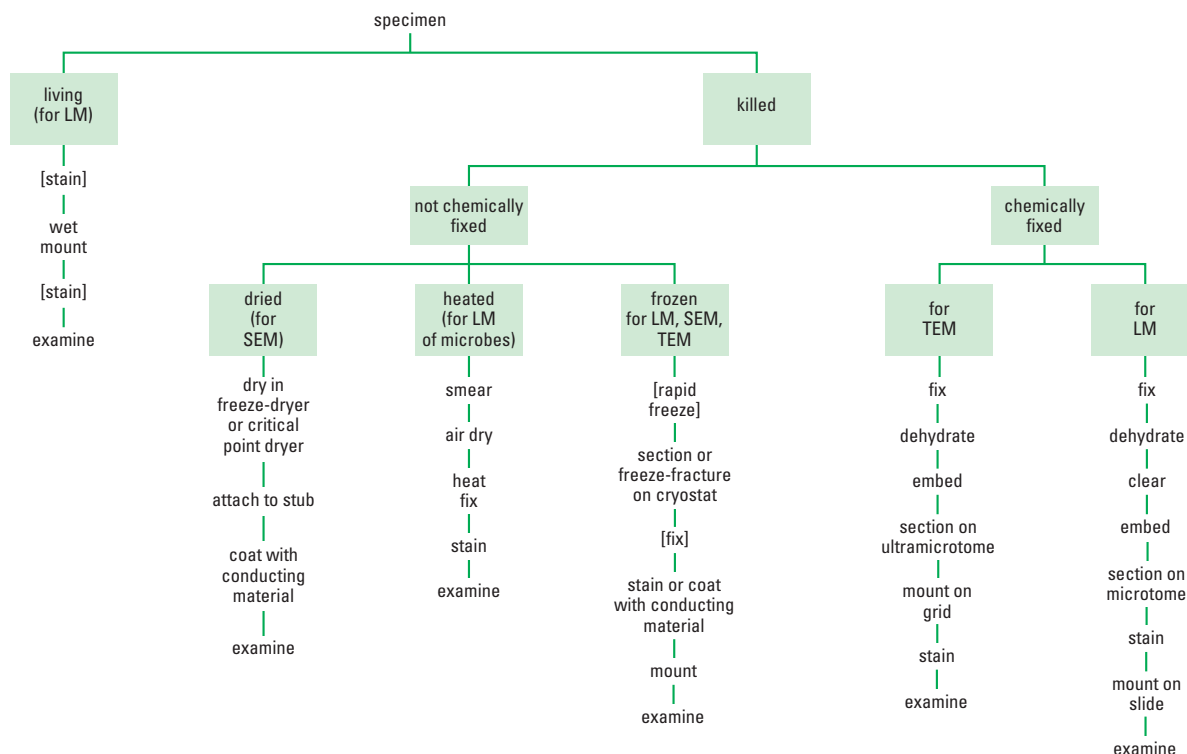


Fig. 25.3 Flowchart of procedures necessary to prepare specimens for different forms of microscopy. Steps enclosed in brackets are optional. LM, light microscope; SEM, scanning electron microscope; TEM, transmission electron microscope.

Preparing specimens

Without careful preparation of the material being studied, the biological structures viewed with any microscope can be rendered meaningless due to artefacts. Figure 25.3 summarises the processes involved for the main types of microscopy discussed above. The processes involved in preparing material for light microscopy are outlined in Chapter 26.

Text reference

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Last accessed 30/04/21.

STUDY EXERCISES

25.1 Test your microscopy knowledge. Indicate whether the following statements about light microscopy, scanning electron microscopy (SEM) or transmission electron microscopy (TEM) are true or false.

- TEM allows you to see at finer resolution than light microscopy.
- TEM allows you to see surface features of specimens.
- SEM always requires staining of specimens.
- The resolution of TEM is about 200 times better than that of light microscopy.
- The resolution of a microscope is linked to the wave-length of electromagnetic radiation employed.
- The specimen in both TEM and SEM is viewed under near-vacuum conditions.
- Specimens for light microscopy can be living or dead.
- SEM provides better resolution than TEM.

- The depth of focus in light microscopy is greater than that in SEM.
- Light microscopy, SEM and TEM all involve the use of a condenser lens within the microscope.

25.2 Fill in the blanks in the following paragraph.

Dark-field microscopy involves shining reflected and _____ light on the specimen against a dark background. It is particularly useful for _____ specimens. UV microscopy uses short-wavelength UV light in order to increase image _____. Phase-contrast microscopy utilises constructive and destructive _____ effects to increase image _____. Nomarski microscopy provides a pseudo _____ image, with a very small depth of _____, allowing _____ to be carried out. _____ light microscopy allows visualisation of optically active components in the specimen. Confocal microscopy involves the use of a _____ light source and can yield computer-generated 3-D images.

STUDY EXERCISES *(continued)*

25.3 Identify the missing preparative procedures. In each sequence below, one or two steps have been missed out. Using Fig. 25.3, identify the missing procedures.

- (a) For light microscopy on a killed and fixed specimen: fix – dehydrate – clear – _____ – section – _____ – mount – examine.
- (b) For light microscopy on a heat-fixed microbial specimen: smear – _____ – heat fix – _____ – examine.
- (c) For TEM on a killed and fixed specimen: fix – _____ – embed – section – mount stain – examine.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

26 Setting up and using a light microscope

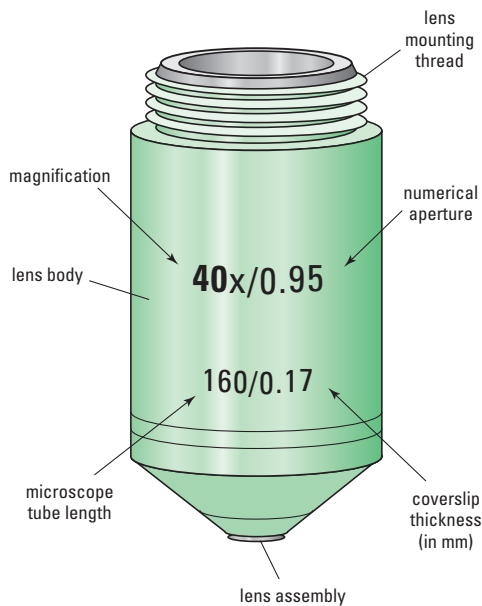


Fig. 26.1 Standard features of an objective lens. Most lenses are inscribed to show the details labelled above. The numerical aperture is a measure of the light-gathering power of the lens.

Using binocular eyepieces – if you do not know your interpupillary distance, ask someone to measure it with a ruler. You should stare at a fixed point in the distance while the measurement is taken (in millimetres). Take a note of the value for future use.

Using microscopes as a spectacle or contact lens wearer – those who wear glasses can remove them for viewing, as microscope adjustments will accommodate most deficiencies in eyesight (except astigmatism). This is more comfortable and stops the spectacle lenses being scratched by the eyepiece holders. However, it may create difficulties in focusing when drawing diagrams. Those with contact lenses should simply wear them as normal for viewing.

The light microscope is one of the most important instruments used in biology practicals and knowing how to use one correctly is a basic and essential skill of biology. A standard undergraduate binocular microscope consists of three main types of optical unit as shown in Fig. 25.1: eyepieces (two), an objective (Fig. 26.1) and a condenser. These are attached to a stand that holds the specimen on a stage (Fig. 26.2). A monocular microscope is constructed similarly but has one eyepiece lens rather than two.

Setting up a binocular light microscope

Before using any microscope, familiarise yourself with its component parts.

The procedures outlined below are simplified to allow you to set up microscopes like those of the Olympus CX series (Fig. 26.2). For monocular microscopes, disregard instructions for adjusting eyepiece lenses (3) and (5).

- 1. Place the microscope at a convenient position on the bench.** Adjust your seating so that you are comfortable operating the focus and stage controls. Unwind the power cable, plug in and switch on after first ensuring that the lamp setting is at a minimum. Then adjust the lamp setting to about two-thirds of the maximum.
- 2. Select a low-power (for example, $\times 10$) objective.** Make sure that the lens clicks home.
- 3. Set the eyepiece (ocular) lenses to your interpupillary distance;** this can usually be read off a scale on the turret. You should now see a single circular field of vision. If you do not, try adjusting in either direction.
- 4. Put a prepared slide on the stage.** Examine it first against a light source and note the position, colour and rough size of the specimen. Place the slide on the stage (coverslip up!) using the slide holder and, viewing from the side, position it with the stage adjustment controls so that the specimen is illuminated.
- 5. Focus the image of the specimen** using first the coarse and then the fine focusing controls (Fig. 26.3). The image will be reversed and upside down compared with that seen when viewing the slide directly.
 - (a) If both eyepiece lenses are adjustable, set your interpupillary distance on the scale on each lens. Close your left eye, look through the right eyepiece with your right eye and focus the image with the normal controls. Now close your right eye, look through the left eyepiece with your left eye and focus the image by rotating the eyepiece holder. Take a note of the setting for future use.
 - (b) If only the left eyepiece is adjustable, close your left eye, look with the right eye through the static right eyepiece and focus the image with the normal controls. Now close your right eye, look through the left eyepiece with your left eye and focus the image by rotating the eyepiece holder. Take a note of the setting for future use.
- 6. Close the condenser–iris diaphragm** (aperture–iris diaphragm), then open it to a position such that further opening has no effect on the brightness of the image (the ‘threshold of darkening’). The edge of the diaphragm should not be in view. Turn down the lamp if it is too bright.

Fig. 26.2 Photograph of a binocular microscope (The Olympus model CX43), showing key features.

- The lamp in the base of the stand (1) supplies light; its brightness is controlled by an on-off switch and voltage control (2). Never use maximum voltage or the life of the bulb will be reduced – a setting two-thirds to three-quarters of maximum should be adequate for most specimens. A field-iris diaphragm may be fitted close to the lamp to control the area of illumination (3).
- The condenser control focuses light from the condenser lens system (4) on to the specimen and projects the specimen's image on to the front lens of the objective. Correctly used, it ensures optimal resolution.
- The condenser-iris diaphragm (5) controls the amount of light entering and leaving the condenser; its aperture can be adjusted using the condenser-iris diaphragm lever (6). Use this to reduce glare and enhance image contrast by cutting down the amount of stray light reaching the objective lens.
- The specimen (normally mounted on a slide) is fitted to a mechanical stage or slide holder using a spring mechanism. Two controls allow you to move the slide in x and y planes. Vernier scales on the slide holder can be used to return to the same place on a slide. The fine and coarse focus controls adjust the height of the stage relative to the lens systems. Take care when adjusting the focus controls to avoid hitting the lenses with the stage or slide.
- The objective lens (9) supplies the initial magnified image; it is the most important component of any microscope because its qualities determine resolution, depth of field and optical aberrations. The objective lenses are attached to a revolving nosepiece (10). Take care not to jam the longer lenses on the stage or slide as you rotate the nosepiece. You should feel a distinct click as each lens is moved into position. The magnification of each objective is written on its side; a normal complement would be $\times 4$, $\times 10$, $\times 40$ and $\times 100$ (oil immersion).
- The eyepiece lens (11) is used to further magnify the image from the objective and to put it in a form and position suitable for viewing. Its magnification is written on the holder (normally $\times 10$). By twisting the holder for one or both of the eyepiece lenses you can adjust their relative heights to take account of optical differences between your eyes. The interpupillary distance scale (12) and adjustment knob allow compensation to be made for differences in the distance between users' pupils.

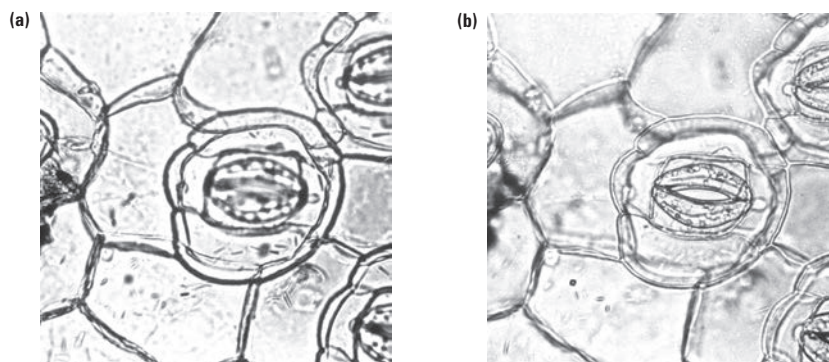
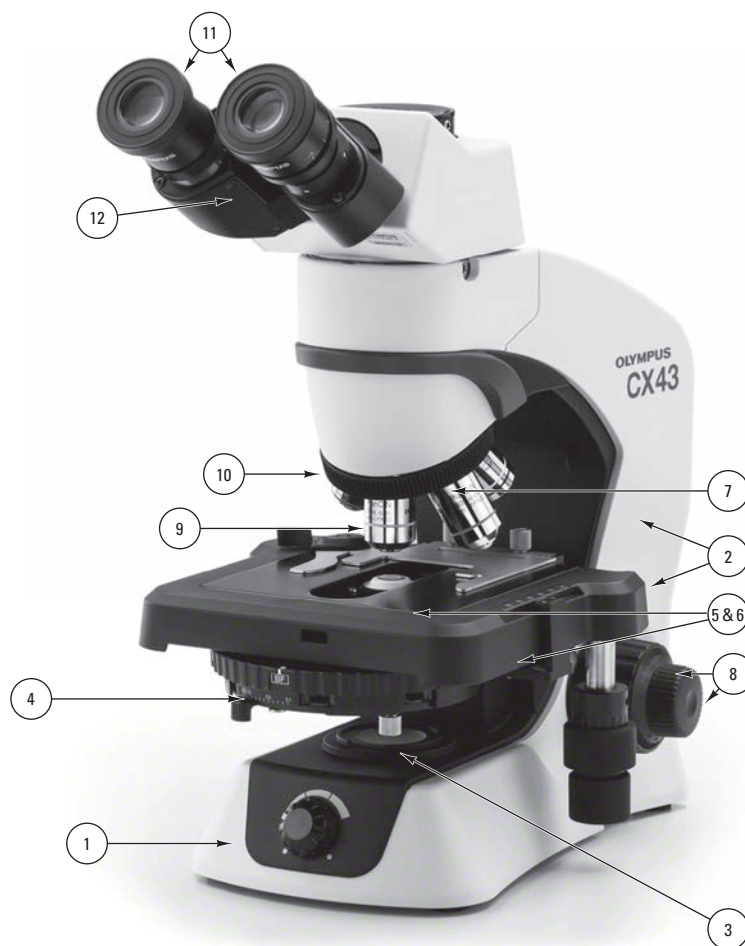


Fig. 26.3 Importance of correct focus in light microscopy. Stomatal complex of *Commelina communis* L., a specimen that is a monolayer of cells approximately 30–50 μm thick. (a) Focal plane is on 'internal' walls of the cells; (b) focal plane is on the 'external' walls and stomatal pore. The two images are different, and while it would not be possible to measure the stomatal pore in (a), it would not be possible to see the vacuolar crystals in (b). When looking at specimens, always use the fine-focus control to view different focal planes.

Adjusting a microscope with a field-iris diaphragm – adjust this before the condenser-iris diaphragm: close it until its image appears in view as a circle of light, if necessary focusing on the edge of the circle with the condenser controls and centring it with the centring screws. Now open it so the whole field is just illuminated.

Using high-power objectives – never remove a slide while a high-power objective lens (i.e. $\times 40$ or $\times 100$) is in position. Always turn back to the $\times 10$ first. Having done this, lower the stage and remove the slide.

7. **Focus the condenser.** Place an opaque pointed object (the tip of a mounted needle or a sharp pencil point) on the centre of the light source. Adjust the condenser setting until both the specimen and needle tip/pencil point are in focus together. Check that the condenser-iris diaphragm is just outside the field of view.
8. **For higher magnifications, swing in the relevant objective** (for example, $\times 40$), carefully checking that there is space for it. Adjust the focus using the fine control only. If the object you wish to view is in the centre of the field with the $\times 10$ objective, it should remain in view (magnified, of course) with the $\times 40$. Adjust the condenser-iris diaphragm and condenser as before – the correct setting for each lens will be different.
9. **When you have finished using the microscope, remove the last slide and clean the stage if necessary.** Turn down the lamp setting to its minimum, then switch off. Clean the eyepiece lenses with lens tissue. Check that the objectives are clean. Unplug the microscope from the mains and wind the cable round the stand and under the stage. Replace the dust cover.

If you have problems in obtaining a satisfactory image, refer to Box 26.1; if this does not help, refer the problem to the class supervisor. Figure 26.4 shows several types of non-biological artefacts that you will come to recognise through practical experience.

KEY POINT Never assume that the previous person to use your microscope has left it set up correctly: apart from taking account of differences in users' eyes, the microscope needs to be properly set up for each lens combination used.

Box 26.1 How to troubleshoot problems in light microscopy

No image; very dark image; image dark and illuminated irregularly

- Microscope not switched on (check plug and base)
- Illumination control at low setting or off
- Objective nosepiece not clicked into place over a lens
- Diaphragm closed down too much or off-centre
- Lamp failure

Image visible and focused but pale and indistinct

- Diaphragm needs to be closed down further (see Fig. 26.5)
- Condenser requires adjustment

Image blurred and cannot be focused

- Dirty objective
- Dirty slide

- Slide upside down
- Slide not completely flat on stage
- Eyepiece lenses not set up properly for user's eyes
- Fine focus at end of travel
- Oil-immersion objective in use, without oil

Dust and dirt in field of view

- Eyepiece lenses dirty
- Objective lens dirty
- Slide dirty
- Dirt on lamp glass or upper condenser lens

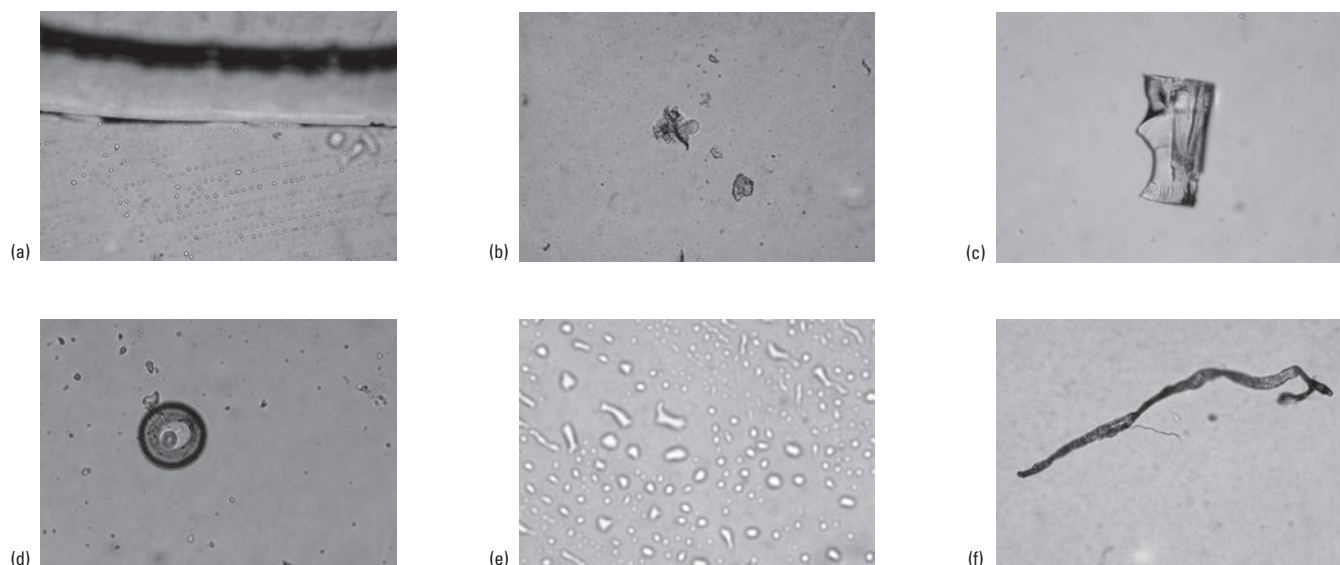


Fig. 26.4 Artefacts in light microscopy. Do not mistake any of the following for biological specimens: (a) edge of coverslip; (b) dust on slide; (c) shard of glass; (d) air bubble; (e) grease spots; (f) cellulose fibre.

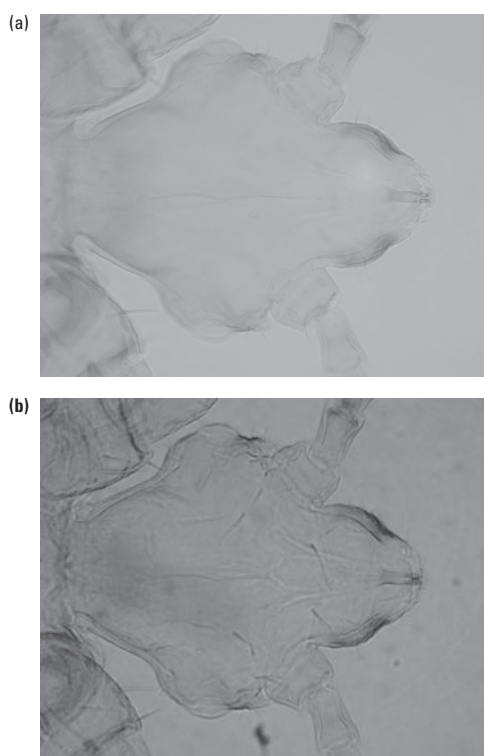


Fig. 26.5 Effect of closing the condenser-iris diaphragm on contrast. Head of human head louse, *Pediculus humanus capitus* DeGeer; (a) with condenser-iris diaphragm open; (b) with condenser-iris diaphragm closed (all other settings the same). Note difference in detail that can be seen in (b), but also that image (b) is darker – when using the condenser-iris diaphragm in this way you may need to compensate by increasing the light setting.

Observing transparent specimens

Some stained preparations and all colourless objects are difficult to see when the microscope is adjusted as below. Contrast can be improved by closing down the condenser-iris diaphragm (Fig. 26.5). Note that when you do this, diffraction haloes appear round the edges of objects. These obscure the image of the true structure of the specimen and may result in loss of resolution. Nevertheless, an image with increased contrast may be easier to interpret.

Using oil-immersion objectives

These provide the highest resolution of which the light microscope is capable. They must be used with immersion oil filling the space between the objective lens and the top of the slide. The oil has the same refractive index as the glass lenses, so loss of light by reflection and refraction at the glass/air interface is reduced. This increases the resolution, brightness and clarity of the image and reduces aberration. Use oil-immersion objective(s) as follows:

1. **Check that the object of interest is in the field of view** using, for example, the $\times 10$ or $\times 40$ objective.
2. **Apply a single small droplet of immersion oil** to the illuminated spot on the top of the slide, having first swung the $\times 40$ objective away. Never use too much oil: it can run off the slide and mess up the microscope.
3. **Move the high-power ($\times 100$) oil-immersion objective into position carefully**, checking first that there is space for it. Focus on the specimen using the fine control only. You may need a higher brightness setting.
4. **Perform condenser-iris diaphragm and condenser focusing adjustments** as for the other lenses.

- 5. When finished, clean the oil-immersion lens** by gently wiping it with clean lens tissue. If the slide is a prepared one, wipe the oil off with lens tissue.

You should take great care when working with oil-immersion lenses as they are expensive to replace. Because the working distance between the lens and coverslip is so short (less than 2 mm), it is easy to damage the lens surface by inadvertently hitting the slide or coverslip surface. You must also remember that they need oil to work properly. If working with an unfamiliar microscope, you can easily recognise oil-immersion lenses. Look for a white or black ring on the lens barrel, near the lens, or for 'oil' clearly marked on the barrel.

Using stains

The purpose of staining in microscopy is to:

- **add contrast to the image**
- **identify chemical components of interest**
- **locate particular tissues, cells or organelles.**

This is achieved in different ways for different types of microscopy. In standard light microscopy, contrast is achieved by staining the structure of interest with a coloured dye; in UV microscopy, contrast is obtained using fluorescent stains. Physico-chemical properties of the stain cause it to attach to certain structures preferentially or be taken up across cell membranes.

Stains for light microscopy are categorised according to the charge on the dye molecule. Stains like haematoxylin, whose coloured part is a cation (i.e. basic dyes), stain acidic, anionic substances like nucleic acids: such structures are termed basophilic. Stains like eosin, whose coloured part is an anion (i.e. acid dyes), stain basic, cationic substances: such structures are termed acidophilic. Acid dyes tend to stain all tissue components, especially at low pH, and are much used as counterstains. Staining is progressive if it results in some structures taking up the dye preferentially. Staining is regressive if it involves initial over-staining followed by decolorisation (differentiation) of those structures that do not bind the dye tightly (for example, Gram staining, pp. 258–9).

Certain 'vital' stains (for example, neutral red) are used to determine cell viability or the pH of cell compartments such as plant vacuoles. 'Mortal' stains (for example, Evans blue) are excluded from living cells but diffuse into dead ones and are used to assay cell mortality.

Table 26.1 gives a number of widely used stains and their application for animal, plant and microbial cells and tissues.

Establishing scale and measuring objects

The magnification of a light microscope image is calculated by multiplying the objective magnification by the eyepiece magnification. However, the magnification of the image bears no certain relation to the magnification of any drawing of the image – you may equally well choose to draw the same image 10 mm or 10 cm long. For this reason, *it is essential to add a scale to all your diagrams*. You can provide either a bar giving the estimated size of an object of interest, or a bar of defined length (e.g. 100 μm).

Table 26.1 A selection of stains for light microscopy of sections

Stain	What it stains	Comments
Plant cells	Chlorazol black	Cell walls: black Nuclei: black, yellow or green Suberin: amber
	Neutral red	Living cells: pink (pH < 7)
	Phloroglucinol/HCl	Lignified cell walls: red
	Ruthenium red	Pectins: red
	Safranin + Fast green	Nuclei, chromosomes, cuticle and lignin: red Other components: green
	Toluidine blue	Lignified cell walls: blue Cellulose cell walls: purple
Fungi and bacteria	Giemsa	Bacterial chromosome: purple Bacterial cytoplasm: colourless
	Gram	Gram-positive bacteria: violet/purple Gram-negative bacteria: red/pink Yeasts: violet/purple
	Grey	Bacterial flagella: red
	Lactophenol cotton blue	Fungal cytoplasm: blue (hyphal wall unstained)
	Nigrosin or India ink	Background: grey-black
	Shaeffer and Fulton	Bacterial endospores: green Vegetative cells: pink/red
	Ziehl-Neelsen	Actinomycetes: red Bacterial endospores: red Other microbes: blue
Animal cells	Azure A/eosin B	Nuclei, RNA: blue Basophilic cells: blue-violet Most other cells: pale blue Muscle cells: pink Necrosing cells: pink Cartilage matrix: red-violet Bone: pink Red blood cells: orange-red Mucins: green-blue/blue-violet
	Chlorazol black	Chitin: greenish-black Nuclei: black, yellow or green Glycogen: pink or red
	Iron haematoxylin	Nuclei, chromosomes and red blood cells: black Other structures: grey or blue-black
	Mallory	Nuclei: red Nucleoli: yellow Collagen, mucus: blue Red blood cells: yellow Cytoplasm: pink or yellow
	Masson's trichrome	Collagen, mucus: green Cytoplasm: orange or pink
	Mayer's (haematoxylin and eosin; 'H&E'; haemalum)	Nuclei: blue/purple Cytoplasm: pink

Using an eyepiece graticule – choose the eyepiece lens corresponding to your stronger eye and check that you have made the correct adjustments to the eyepiece lenses as detailed on p. 175.

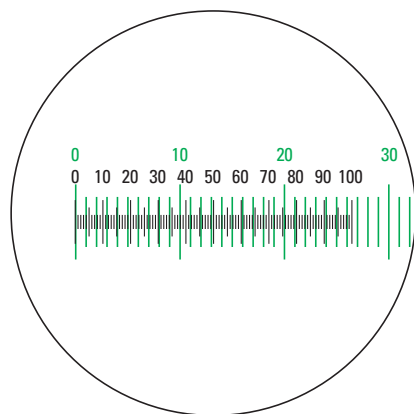


Fig. 26.6 Calibrating an eyepiece micrometer (graticule). Align two scales and read the number of stage micrometre divisions (shown in colour) for a particular number of eyepiece micrometre divisions (shown in black). In this case 26.5 scale divisions of 0.01 mm are equivalent to 100 eyepiece divisions, so each eyepiece division $0.265 \div 100 = 0.00265 \text{ mm} = 2.65 \mu\text{m}$. This is a typical value for a total magnification of $\times 400$ (e.g. $\times 40$ objective and $\times 10$ eyepiece).

SAFETY NOTE Take care when moving microscopes, not only because of the cost of replacement if damaged, but also because they weigh several kilograms and could cause injury if dropped. Always carry a microscope using two hands.

Measuring specimens using a dissecting microscope – because of the low magnification, sizes can generally be estimated by comparison with a ruler placed alongside the specimen. If accurate measurements are required, eyepiece graticules can be used (Fig. 46.2).

The simplest method of estimating linear dimensions is to compare the size of the image to the diameter of the field of view. You can make a rough estimate of the field diameter by focusing on the millimeter scale of a transparent ruler using the lowest power objective. Estimate the diameter of this field directly, then use the information to work out the field diameters at the higher powers *pro rata*. For example, if the field at an overall magnification of $\times 40$ is 4 mm, at an overall magnification of $\times 100$ it will be: $40/100 \times 4 \text{ mm} = 1.6 \text{ mm}$ (1600 μm).

Greater accuracy can be obtained if an eyepiece micrometer (graticule) is used. This carries a fine scale and fits inside an eyepiece lens. The eyepiece micrometer is calibrated using a stage micrometer, basically a slide with a fine scale on it.

Figure 26.6 shows how to calibrate an eyepiece micrometer, along with a worked example. Once you have calibrated your eyepiece micrometer for each objective lens used, you can use it to measure objects: in the example shown in Fig. 26.6, the scale reading is multiplied by 2.65 μm to give the value in micrometers. So, if you measured the width of a human hair at 34 eyepiece micrometer units, then this will be equal to $34 \times 2.65 = 90.1 \mu\text{m}$. An alternative approach is to put a scale bar on a diagram; for example, a 100 μm scale bar would be equivalent to the length of almost 38 eyepiece micrometer divisions. You should avoid putting too many significant figures in any estimates of dimensions: there may be quite large errors involved, which could make the implied accuracy misleading (spurious accuracy, p. 189).

Caring for your microscope

Microscopes are delicate, precision instruments. Handle them with care and never force any of the controls. Do not touch any of the glass surfaces with anything other than clean, dry lens tissue. Bear in mind that a replacement would be very expensive.

If moving a microscope, hold the stand above the stage with one hand and rest the base of the stand on your other hand. Always keep the microscope vertical, or the eyepieces may fall out. Put the microscope down gently.

Clean lenses by gently wiping with clean, dry lens tissue. Use each piece of tissue once only. Try not to touch lenses with your fingers as oily fingerprints are difficult to clean off. Do not allow any solvent (including water) to come into contact with a lens; sea water is particularly damaging.

Using a dissecting (stereoscopic) microscope

This form of stereoscopic microscope (Fig. 26.7) is used for observations at low total magnification ($\times 4$ to $\times 50$) where a large working distance between objectives and stage is required, perhaps because the specimen is not flat or dissecting instruments are to be used. A stereoscopic microscope essentially consists of two separate lens systems, one for each eye. Some instruments incorporate zoom objectives. The eyepiece-objective combinations are inclined at about 15° to each other and the brain resolves the compound image in three dimensions as it does for normal vision. The image is right side up and not reversed, which is ideal for dissections. Specimens are often viewed in a fresh state and need not be placed on a slide – they might be in

a Petri dish or on a white tile. Illumination can be from above or below the specimen, as desired.

Most of the instructions for the binocular microscope given above apply equally well to dissecting microscopes, although the latter do not normally have adjustable condensers or diaphragms. With stereoscopic microscopes, make specially sure to adjust the eyepiece lenses to suit your eyes so that you can take full advantage of the stereoscopic effect.

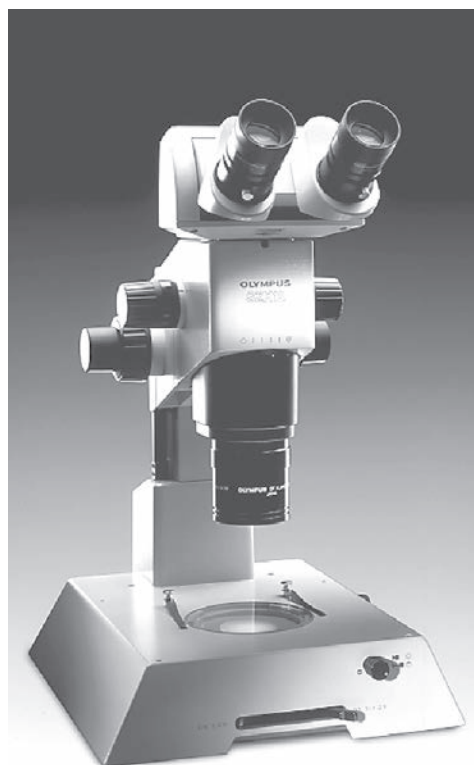


Fig. 26.7 A photograph of a typical microscope (the Olympus Model SZX12), published courtesy of Olympus Optical Company (UK) Ltd.

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STUDY EXERCISES

26.1 Test your knowledge of the parts of a binocular light microscope. Cover up the legend on the left of Fig. 26.2 with a piece of paper or card. Now identify the parts of the light microscope numbered on the diagram. Check your answers from the legend.

26.2 Identify roles of parts of a binocular light microscope. State briefly the *primary* role of each component of a standard binocular light microscope:

- (a) condenser
- (b) objective lens
- (c) condenser-iris diaphragm
- (d) interpupillary distance scale
- (e) Vernier scales on the mechanical stage.

26.3 Identify the correct sequence of adjustments when setting up a light microscope.

- (a) Focus specimen – set interpupillary distance – adjust condenser-iris diaphragm – make individual

eyepiece adjustment – focus condenser – focus specimen.

- (b) Make individual eyepiece adjustment – set interpupillary distance – focus condenser – focus specimen – adjust condenser-iris diaphragm – focus specimen.
- (c) Set interpupillary distance – focus specimen – make individual eyepiece adjustment – adjust condenser-iris diaphragm – focus condenser – focus specimen.
- (d) Make individual eyepiece adjustment – set interpupillary distance – focus specimen – adjust condenser-iris diaphragm – focus condenser – focus specimen.
- (e) Focus specimen – make individual eyepiece adjustment – set interpupillary distance – focus condenser – adjust condenser-iris diaphragm.

Answers to these study exercises are available at go.pearson.com/uk/he/resources



The investigative approach

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27 Making measurements

Definition

Variable – any characteristic or property that can take one of a range of values (contrast this definition with that for a *parameter*, which is a numerical constant in any particular instance).

Working with discontinuous variables – note that while the original data values must be integers, derived data and statistical values do not have to be whole numbers. Thus, it is perfectly acceptable to express the *mean* number of colonies per Petri plate as 24.6.

The term data (singular = datum, or data value) refers to items of information, and you will use different types of data from a wide range of sources during your practical work. Consequently, it is important to appreciate the underlying features of data collection and measurement.

Quantifying variables

Biological variables (Fig. 27.1) can be classified as follows:

Quantitative variables

These are characteristics whose differing states can be described by means of a number. They are of two basic types:

- 1. Continuous variables**, such as length; these are usually measured against a numerical scale. Theoretically, they can take any value on the measurement scale. In practice, the number of significant figures of a measurement is directly related to the precision of your measuring system (p. 189); for example, dimensions measured with Vernier calipers will provide readings of greater precision than a millimetre ruler. Many of the variables measured in biomolecular sciences are continuous, for example mass, temperature, time, amount of produce formed by an enzyme.
- 2. Discontinuous (discrete) variables**, such as the number of bacterial colonies on a Petri plate; these are always obtained by counting and therefore the data values must be whole numbers (integers). There are no intermediate values.

Ranked variables

These provide data that can be listed in order of magnitude (i.e. ranked). A familiar example is the abundance of an organism in a sample, which is often expressed as a series of ranks, for example, rare = 1, occasional = 2, frequent = 3, common = 4 and abundant = 5. When such data are given numerical ranks, rather than descriptive terms, they are sometimes called ‘semi-quantitative data’. Note that the difference in magnitude between ranks need not be consistent. For example, regardless of whether there was a one-year or a five-year gap between offspring in a family, their rank in order of birth would be the same.

Qualitative variables (attributes)

These are non-numerical and descriptive; they have no order of preference, and therefore are not measured on a numerical scale nor ranked in order of magnitude, but are described in terms of categories. Examples include viability (i.e. dead or alive), shape (for example, round, flat, elongated, etc.) and presence/absence of a particular biomolecule in a sample (p. 423).

Variables may be independent or dependent. Usually, the variable under the control of the experimenter (for example, time) is the independent variable, while the variable being measured (for example, rate of a reaction) is the dependent variable (p. 200). Sometimes it is not appropriate to describe variables in this way, and they are then referred to as interdependent variables (for example, the length and breadth of an organism).

The majority of data values are recorded as direct measurements, readings or counts, but there is an important group, called derived (or

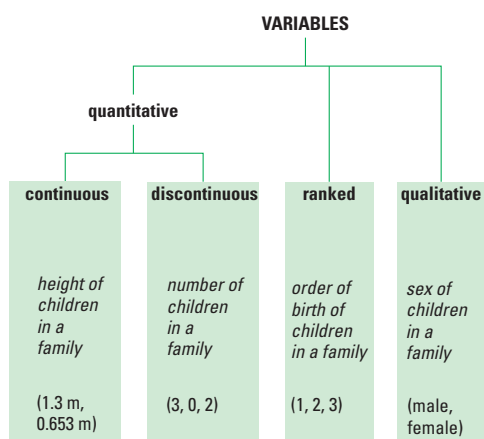


Fig. 27.1 Examples of the different types of variables as used to describe some characteristics of families.

Examples A **nominal scale** for temperature is not feasible, since the relevant descriptive terms can be ranked in order of magnitude.

An **ordinal scale** for temperature measurement might use descriptive terms, ranked in ascending order, e.g. cold = 1, cool = 2, warm = 3, hot = 4.

The **Celsius scale** is an interval scale for temperature measurement, since the arbitrary zero corresponds to the freezing point of water (0 °C).

The **kelvin scale** is a ratio scale for temperature measurement since 0 K represents a temperature of absolute zero (for information, the freezing point of water is 253.15 K on this scale).

computed), that results from calculations based on two or more data values; for example, ratios, percentages, indices and rates.

Using measurement scales

Variables may be measured on different types of scale:

- **Nominal scale:** this classifies objects into categories based on a descriptive characteristic. It is the only scale suitable for qualitative data.
- **Ordinal scale:** this classifies by rank. There is a logical order in any number scale used.
- **Interval scale:** this is used for quantitative variables. Numbers on an equal-unit scale are related to an arbitrary zero point.
- **Ratio scale:** this is similar to the interval scale, except that the zero point now represents an absence of that character (i.e. it is an absolute zero). In contrast to the interval scale, the ratio of two values is meaningful (for example, a temperature of 200 K is twice that of 100 K, but a temperature of 20 °C is not twice that of 10 °C).

The measurement scale is important in determining the mathematical and statistical methods used to analyse your data. Table 27.1 presents a summary of the important properties of these scales. Note that you may be

Table 27.1 Some important features of scales of measurement

	Measurement scale			
	Nominal	Ordinal	Interval	Ratio
Type of variable	Qualitative (Ranked)* (Quantitative)*	Ranked (Quantitative)*	Quantitative	Quantitative
Examples	Species Sex Colour	Abundance scales Reproductive condition Optical assessment of colour development	Fahrenheit temperature scale Date (BC/AD)	Kelvin temperature scale Weight Length Response time Most physical measurements
Mathematical properties	Identity	Identity Magnitude	Identity Magnitude Equal intervals	Identity Magnitude Equal intervals True zero point
Mathematical operations possible on data	None	Rank	Rank Addition Subtraction	Rank Addition Subtraction Multiplication Division
Typical statistics used	Only those based on frequency of counts made: contingency tables, frequency distributions, etc. Chi- square test	Non-parametric methods, sign tests Mann-Whitney <i>U</i> -test	Almost all types of test, <i>t</i> -test, analysis of variance (ANOVA), etc. (check distribution before using, Chapter 77)	Almost all types of test, <i>t</i> -test, ANOVA, etc. (check distribution before using, Chapter 77)

*In some instances (see text for examples).

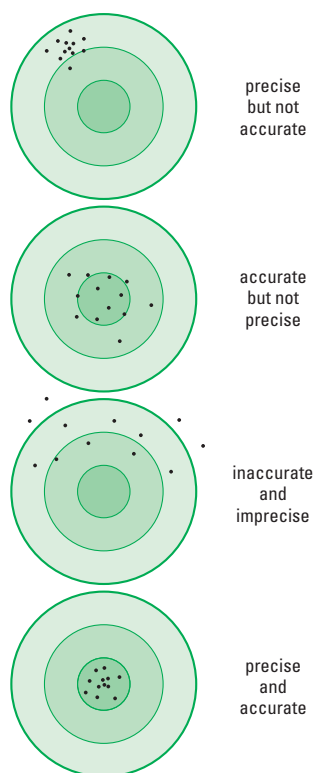


Fig. 27.2 'Target' diagrams illustrating precision and accuracy.

able to measure a characteristic in more than one way, or you may be able to convert data collected in one form to a different form. For instance, you might measure light in terms of the photon flux density (p. 328) between particular wavelengths of the EMR spectrum (ratio scale), or simply as 'blue' or 'red' (nominal scale); you could find out the dates of birth of individuals (interval scale) but then use this information to rank them in order of birth (ordinal scale). Where there are no other constraints, you should use a ratio scale to measure a quantitative variable, since this will allow you to use the broadest range of mathematical and statistical procedures (Table 27.1).

Determining accuracy and precision

Accuracy is the closeness of a measured or derived data value to its true value, while precision is the closeness of repeated measurements to each other (Fig. 27.2). A balance with a fault in it (i.e. a bias, see below) could give precise (i.e. very repeatable) but inaccurate (i.e. untrue) results. Unless there is bias in a measuring system, precision will lead to accuracy and it is precision that is generally the most important practical consideration, if there is no reason to suspect bias. You can investigate the precision of any measuring system by repeated measurements of individual samples, while you can investigate the accuracy of any measuring system by measuring a 'standard' of known value (Chapter 43).

Absolute accuracy and precision are impossible to achieve, due to both the limitations of measuring systems for continuous quantitative data and the fact that you are usually working with incomplete data sets (samples). It is particularly important to avoid *spurious accuracy* in the presentation of results; include only those digits that the accuracy of the measuring system implies. This type of error is common when changing units (for example, inches to metres) and in derived data, especially when calculators give results to a large number of decimal places. Further advice is given on p. 582.

Monitoring bias (systematic error) and consistency

Bias is a consistent non-random divergence from accuracy and is one of the most troublesome difficulties in using numerical data. Biases may be associated with incorrectly calibrated instruments (for example, a faulty pipettor, or with experimental manipulations, for example, shrinkage during the preservation of a specimen). Bias in measurement can also be subjective, or personal, for example, an experimenter's preconceived ideas about an 'expected' result.

Bias can be minimised by using a carefully standardised procedure, with fully calibrated instruments. You can investigate bias in 'trial runs' by measuring the same variable in several different ways, to see whether the same result is obtained.

To avoid personal bias, 'blind' measurements should be made where the identity of individual samples is not known to the operator, for example, using a coding system.

Estimating measurement error

All measurements are subject to error, but the dangers of misinterpretation are reduced by recognising and understanding the likely sources of error and by adopting appropriate protocols and calculation procedures.

Minimising errors – determine early in your study what the dominant errors are likely to be and concentrate your time and effort on reducing these.

Working with derived data – special effort should be made to reduce measurement errors because their effects can be magnified when differences, ratios, indices or rates are calculated.

A common source of measurement error is carelessness, for example, reading a scale in the wrong direction or parallax errors (see Fig. 22.1). This can be reduced greatly by careful recording and in some cases may be detected by repeating the measurement. Other errors arise from faulty or inaccurate equipment, but even a perfectly functioning machine has distinct limits to the accuracy and precision of its measurements. These limits are often quoted in manufacturers' specifications and are applicable when an instrument is new; however, you should allow for some deterioration with age.

Further errors are introduced when measurements may be subject to influences outside your control. Resolving such problems requires appropriate experimental design and sampling procedures (Chapter 29). One major influence virtually impossible to eliminate is the effect of the investigation itself: even putting a thermometer in a liquid may change the temperature of the liquid. The very act of measurement may give rise to a confounding variable (p. 201) as discussed in Chapter 29.

KEY POINT You should include descriptions of possible sources of error and estimates of their likely importance in any report. However, do not use 'biological variability' as a catch-all excuse for poor technique or inadequacies in your experimental design.

Sources for further study

Anon. *Measurement*. Available: <https://en.wikipedia.org/wiki/Measurement> Last accessed 11/03/21.

Erikson, B.H. and Nosanchuk, T.A. (1992) *Understanding Data*, 2nd edn. Open University Press, Milton Keynes. [A text aimed at social science students but with clear explanations of issues that are generic, including information on analysis of data.]

Shuttleworth, M. *Choosing Scientific Measurements*. Available <https://explorable.com/scientific-measurements> Last accessed 11/03/21.

STUDY EXERCISES

27.1 Classify variables. Decide on the type of variables used for the following measures, indicating whether they are quantitative or qualitative, continuous or discontinuous, and the type of scale that would be used.

- (a) Number of organisms in a population.
- (b) Length of individuals in a population.
- (c) Colour of flowers.
- (d) Species present in a sample.
- (e) Date of a sample.
- (f) Reproductive status of an animal.

27.2 Investigate types of errors. A student weighed a set of standard masses on two electronic balances and obtained the readings shown in the table below. Explain these results in terms of the type of error involved in each case.

Comparison of weights of masses on two balances					
	Standard mass (g)				
	10	25	50	100	250
Reading (balance A)	10.050	25.049	50.051	100.048	250.052
Reading (balance B)	10.004	25.011	50.021	100.039	250.102

27.3 Investigate methods for carrying out 'blind' measurements. Using online sources, write a description (no more than 250 words) of the approach generally used to carry out 'blind' studies. Your description should define the terms 'single-blind', 'double-blind' and 'triple-blind'; the process of 'unblinding' and the benefits of these processes for scientific study.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

28 Understanding SI units and their use

Accounting for dimensionless measurements – some quantities can be expressed as dimensionless ratios or logarithms (e.g. pH), and in these cases you do not need to use a qualifying unit.

Table 28.1 SI base units

Measured quantity	Name of SI unit	Symbol
Base units		
Length	metre	m
Mass	kilogram	kg
Amount of substance	mole	mol
Time	second	s
Electric current	ampere	A
Temperature	kelvin	K
Luminous intensity	candela	Cd

Table 28.3 SI prefixes

Multiple	Prefix	Symbol	Multiple	Prefix	Symbol
10^{-1}	deci	d	10^1	deca	Da
10^{-2}	centi	c	10^2	hecto	h
10^{-3}	milli	m	10^3	kilo	k
10^{-6}	Micro	μ	10^6	Mega	M
10^{-9}	Nano	n	10^9	Giga	G
10^{-12}	Pico	p	10^{12}	Tera	T
10^{-15}	Femto	f	10^{15}	Peta	P
10^{-18}	Atto	a	10^{18}	Exa	E
10^{-21}	Zepto	z	10^{21}	Zetta	Z
10^{-24}	Yocto	y	10^{24}	Yotta	Y

The System International d'Unités (SI) is the internationally ratified form of the metre-kilogram-second system, providing a system of measurement, based on physical constants. This single unified system is essential for appropriate acquisition of data and efficient communication across the scientific community. Consequently, it is important to understand how SI operates in practice, and how SI relates to other systems of measurement.

Following the principles of the SI

Whenever you make a measurement, you normally state both a number and a unit (for example, 'the length is 1.85 metres'). The number expresses the ratio of the measured quantity to a fixed standard, while the unit identifies that standard measure or dimension. In SI, the units are defined according to fixed standards, specified in 2018 in terms of physical constants of nature (for background, consult the *SI Brochure*, BIPM 2019). While some aspects of SI can seem complex and the scale of some units can seem inconvenient, you will gain most by observing its conventions, particularly in relation to complex calculations where you may be dealing with several measured quantities (pp. 581). The system comprises:

- **seven base units**, each being defined in terms of physical constants and each with a specified abbreviation or symbol (Table 28.1)
- **derived units**, obtained from combinations of base units, which may also be given special symbols (Table 28.2)
- **a set of prefixes** to denote multiplication factors from 10^{-24} to 10^{24} , used for convenience to express multiples or fractions of units (Table 28.3).

Table 28.2 Some important derived SI units

Measured quantity	Name of unit	Symbol	Definition in base units	Alternative in derived units
Energy	Joule	J	$\text{m}^2 \text{kg s}^{-2}$	N m
Force	newton	N	m kg s^{-2}	J m^{-1}
Pressure	Pascal	Pa	$\text{kg m}^{-1} \text{s}^{-2}$	N m^{-2}
Power	Watt	W	$\text{m}^2 \text{kg s}^{-3}$	J s^{-1}
Electric charge	coulomb	C	As	J V^{-1}
Electric potential difference	Volt	V	$\text{m}^2 \text{kg A}^{-1} \text{s}^{-3}$	J C^{-1}
Electric resistance	Ohm	Ω	$\text{m}^2 \text{kg A}^{-2} \text{s}^{-3}$	VA^{-1}
Electric conductance	siemens	S	$\text{s}^3 \text{A}^2 \text{kg}^{-1} \text{m}^{-2}$	A V^{-1} or Ω^{-1}
Electric capacitance	Farad	F	$\text{s}^4 \text{A}^2 \text{kg}^{-1} \text{m}^{-2}$	C V^{-1}
Luminous flux	Lumen	lm	cd sr	
Illumination	Lux	lx	cd sr m^{-2}	lm m^{-2}
Frequency	Hertz	Hz	s^{-1}	
Radioactivity	becquerel	Bq	s^{-1}	
Enzyme activity	katal	kat	$\text{mol substrate s}^{-1}$	

Example 10 μm is correct, while 10 μm , 10 μm . and 10 μ m are incorrect.

Example 2.6 mol is right, but 2.6 mols and 2.6 mol. are wrong.

Example n stands for nano and N for newtons.

Example 1982 963.192 309 kg (perhaps better expressed as 1.982 963 192 309 Gg).

Example 10 m s^{-1} and 10 $\text{m} \cdot \text{s}^{-1}$ are correct, while 10 ms^{-1} and 10 m.s^{-1} are incorrect.

Example milligram and mg are correct, microkilogram and μkg are incorrect.

Examples 10 μm is preferred to 0.000 01 m or 0.010 mm.

1 $\text{mm}^2 = 10^{-6} \text{m}^2$ (not one-thousandth of a square metre).

1 dm^3 (1 L) is more properly expressed as $1 \times 10^{-3} \text{m}^3$.

The mass of a neutrino is 10^{-36}kg .

State as MW m^{-2} rather than W mm^{-2} .

Describing measurements in SI units

Basic format

- **Express each measurement as a number separated from its units by a space.** If a prefix is required, no space is left between the prefix and the unit it refers to.
- **Symbols for units are written in their singular form;** they do not require decimal points to show that they are abbreviated.
- **Give symbols and prefixes appropriate upper-case or lower-case initial letters** as this may define their meaning. Upper-case symbols are named after persons but when written out in full they are not given initial capital letters.
- **Show the decimal sign as a full point on the line.** Some metric countries continue to use the comma for this purpose and you may come across this in the literature: therefore, commas should *not* be used to separate groups of thousands. In numbers that contain many significant figures, you should separate multiples of 10^3 by spaces rather than commas.

Compound expressions for derived units

- **Use either a space, or a ‘half-high’ (centred) dot (·) to separate units in compound expressions** to avoid the potential for confusion with prefixes – contrast ‘ms’ (millisecond) with ‘m s’ (metre-second). Do not use a decimal point on the line within compound units.
- **Express compound units by using negative powers** rather than a solidus (/): for example, write mol m^{-3} rather than mol/m^3 . The solidus is reserved for separating a descriptive label from its units (see p. 566).
- **Use parentheses to enclose expressions being raised to a power** if this avoids confusion: for example, a photosynthetic rate might be given in $\text{mol CO}_2(\text{mol photons})^{-1} \text{s}^{-1}$.
- **Where there is a choice, select relevant (natural) combinations of derived and base units:** for example, you might choose units of Pa m^{-1} to describe a hydrostatic pressure gradient rather than $\text{kg m}^{-2} \text{s}^{-2}$, even though these units are equivalent and the measurements are numerically the same.

Use of prefixes

- **Use prefixes to denote multiples of 10** (Table 28.3) so that numbers are kept between 0.1 and 1000. Express very large or very small numbers as a number between 1 and 10 multiplied by a power of 10 if they are outside the range of prefixes shown in Table 28.3.
- **Treat a combination of a prefix and a symbol as a single symbol.** Thus, when a modified unit is raised to a power, this refers to the whole unit including the prefix.
- **Do not use prefixes in the middle of derived units:** prefixes should be attached only to a unit in the numerator.
- **Use prefix names and symbols for mass based on gram (g)** rather than kilogram (kg), since kg is the only SI base unit that includes a prefix.

Box 28.1 How to interconvert values between some non-SI units and the SI

Quantity	SI unit/symbol	Old unit/symbol	Multiply number in non-SI unit by this factor for equivalent in SI unit*	Multiply number in SI unit by this factor for equivalent in non-SI unit*
Area	square metre/m ²	acre	$4.046\,86 \times 10^3$	$0.247\,105 \times 10^{-3}$
		hectare/ha	10×10^3	0.1×10^{-3}
		square foot/ft ²	0.092 903	10.763 9
		square inch/in ²	645.16×10^{-9}	$1.550\,00 \times 10^6$
		square yard/yd ²	0.836 127	1.195 99
Angle	radian/rad	degree/°	$17.453\,2 \times 10^{-3}$	57.295 8
Energy	joule/J	erg	0.1×10^{-6}	10×10^6
		kilowatt hour/kWh	3.6×10^6	$0.277\,778 \times 10^{-6}$
		calorie/cal	4.186 8	0.238 8
Length	metre/m	Ångstrom/Å	0.1×10^{-9}	10×10^9
		foot/ft	0.304 8	3.260 84
		inch/in	25.4×10^{-3}	39.370 1
		mile	$1.609\,34 \times 10^3$	$0.621\,373 \times 10^{-3}$
		yard/yd	0.914 4	1.093 61
Mass	kilogram/kg	ounce/oz	$26.349\,5 \times 10^{-3}$	35.274 0
		pound/lb	0.453 592	2.204 62
		stone	6.350 29	0.157 473
		hundredweight/cwt	50.802 4	$19.684\,1 \times 10^{-3}$
		ton (UK)	$1.016\,05 \times 10^3$	$0.984\,203 \times 10^{-3}$
Pressure	pascal/Pa	atmosphere/atm	101 325	$9.869\,23 \times 10^{-6}$
		bar/b	100 000	10×10^{-6}
		millimetre of mercury/mmHg	133.322	$7.500\,64 \times 10^{-3}$
		torr/Torr	133.322	$7.500\,64 \times 10^{-3}$
Radioactivity	becquerel/Bq	curie/Ci	37×10^9	$27.027\,0 \times 10^{-12}$
Temperature	kelvin/K	centigrade (Celsius)		
		degree/°C	°C + 273.15	K – 273.15
		Fahrenheit degree/°F	(°F + 459.67) × 5/9	(K × 9/5) – 459.67
Volume	cubic metre/m ³	cubic foot/ft ³	0.026 316 8	35.314 7
		cubic inch/in ³	$16.387\,1 \times 10^{-6}$	$61.023\,6 \times 10^3$
		cubic yard/yd ³	0.764 555	1.307 95
		UK pint/pt	$0.568\,261 \times 10^{-3}$	1 759.75
		US pint/liq pt	$0.473\,176 \times 10^{-3}$	2 113.38
		UK gallon/gal	$4.546\,09 \times 10^{-3}$	219.969
		US gallon/gal	$3.785\,41 \times 10^{-3}$	264.172

*In the case of temperature measurements, use formulae shown.

Expressing units of volume – in this book we use the symbol ‘L’ rather than ‘l’ for litre to avoid potential confusion between ‘i’ and ‘l’. We use SI units where this simplifies calculations. In formal scientific writing, constructions such as $1 \times 10^{-6} \text{ m}^3$ (= 1 mL) and 1 mm^3 (= 1 μL) may be used.

Dealing with the implications of SI in biology

Volume

The SI unit of volume is the cubic metre, m³ (US spelling ‘meter’), which is rather large for practical purposes. The litre (US spelling ‘liter’) is a non-SI unit, equivalent to 1 dm³ or 10⁻³ m³ in SI terms. However, the litre is widely used by many working scientists and glassware is still calibrated in litres. You may find litre given the symbol L, as in this text. SI prefixes can be used, for example, mL for millilitre. However, when such units are used, some of the advantages of the SI are lost, for example, in complex calculations with compound units, p. 581.

Table 28.4 Some physical constants in SI terms

Physical constant	Symbol	Value and units
Avogadro's constant	N_A	$6.022\,174 \times 10^{23} \text{ mol}^{-1}$
Boltzmann's constant	K	$1.380\,626 \times 10^{-23}$
Charge of electron	E	$1.602\,192 \times 10^{-19} \text{ C}$
Gas constant	R	$8.314\,43 \text{ J K}^{-1} \text{ mol}^{-1}$
Faraday's constant	F	$9.648\,675 \times 10^4 \text{ C mol}^{-1}$
Molar volume of ideal gas at STP	V_0	$0.022\,414 \text{ m}^3 \text{ mol}^{-1}$
Speed of light <i>in vacuo</i>	C	$2.997\,924 \times 10^8 \text{ ms}^{-1}$
Planck's constant	H	$6.626\,205 \times 10^{-34} \text{ J s}$

Expressing enzyme activity – the derived SI unit is the katal (kat), which is the amount of enzyme that will transform 1 mol of substrate in 1 s (see Chapter 62).

Mass

The SI unit for mass is the kilogram (kg) rather than the gram (g): this is unusual because the base unit has a prefix applied, for historical reasons (BIPM, 2019). However, you should use prefixes with gram (g), not kilogram (kg).

Amount of substance

You should use the mole (mol, i.e. Avogadro's constant, see Table 28.4). The mole gives the number of specified elementary entities – atoms, molecules, ions or elementary particles. Always specify the elementary particles referred to in other situations (for example, mol photons $\text{m}^{-2} \text{ s}^{-1}$).

Converting between concentration units – being able to express concentrations in different units is important, as this skill is frequently used when following instructions and interpreting data (Chapter 23).

Concentration

The SI unit of concentration, mol m^{-3} , is quite convenient for biological systems. It is equivalent to the non-SI term 'millimolar' ($\text{mM} = \text{mmol L}^{-1}$), while 'molar' ($\text{M} = \text{mol L}^{-1}$) becomes mol dm^{-3} or kmol m^{-3} . Note that the symbol M in the SI is reserved for mega and hence should not be used for concentrations. If the solvent is not specified, then it is assumed to be water (see Chapter 23).

Time

In general, use the second (s) when reporting physical quantities having a time element (for example, give photosynthetic rates in $\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). Hours (h), days (d) and years are acceptable non-SI units and should be used if seconds are clearly absurd (for example, samples were taken over a 5-year period). Note, however, that you may have to convert these units to seconds when doing calculations.

Temperature

The SI unit is the kelvin, K. The non-SI 'degree Celsius' scale has units of the same magnitude, $^{\circ}\text{C}$, but starts at 273.15K, the melting point of ice at standard temperature and pressure (293.15 K and 0.101 325 MPa). Temperature is similar to time in that the Celsius scale is in widespread use, but note that conversions to K may be required for calculations. Note also that you must *not* use the degree sign ($^{\circ}$) with K and that this symbol must be in upper case to avoid confusion with k for kilo; however, you *should* retain the degree sign with $^{\circ}\text{C}$ to avoid confusion with the coulomb, C.

Accounting for unit oddities – examples of nomenclature that do not fit easily into the SI:

- **Dates.** The UK convention is 10/03/19 for 10 March 2019, but in the US this would mean 3 October 2019. Avoid confusion by stating 3 Mar 2019 or making it clear which convention you are adopting in your Materials and Methods or table/figure footnotes/legend.
- **Ohm.** If the symbol Ω is not available in your word processor, then write 'ohm' (not capital O).
- **Computing terminology.** b is used for bit, and B for Byte. Mega and giga can be ambiguous as they may refer loosely to 2^{20} or 2^{30} respectively for binary data.

Electromagnetic radiation (EMR)

This has the characteristics of a particle and of a vibrating wave, travelling in discrete particulate units, termed 'photons'. Consequently, EMR can be measured in terms of the number of photons (mol), or power (W). This is considered further in Chapter 45 (p. 326) in relation to the measurement of light – that part of the EMR spectrum detected by the human eye.

KEY POINT For the foreseeable future, you will need to make conversions from other units to SI units, as much of the literature quotes data using imperial, centimetre-gram-second (cgs) or other systems. You will need to recognise these units and find the conversion factors required. Examples relevant to biology are given in Box 28.1. Table 28.4 provides values of some important physical constants in SI units.

Text reference and source for further study

BIPM – Bureau International des Poids et Mesures (2019) *SI Brochure: The International System of Units (SI)*. Available: <https://www.bipm.org/en/publications/si-brochure/> Last accessed 11/03/21.

STUDY EXERCISES

28.1 Practise converting between units. Using Box 28.1 as a source, convert the following amounts into the units shown. Give your answers to three significant figures.

- 101 000 Pa into atmospheres.
- One square yard into square millimetres.
- One UK pint into millilitres.
- 37 °C into Kelvin.
- 11 stone 6 pounds into kilograms.

28.2 Practise using prefixes appropriately. Simplify the following number/unit combinations using an appropriate prefix so that the number component lies between 0.1 and 1000.

- 10 000 mm
- 0.015 mL
- $5 : 10^9$ J
- 65 000 ms⁻¹
- 0.000 000 0001 g

28.3 Check units in an equation. The Hagen–Poiseuille equation describes water flux in smooth cylindrical pipes assuming laminar flow. This equation can be expressed as:

$$J_v = \frac{r^2 \times \delta P}{8\eta \times \delta x}$$

where:

r = the radius of the cylindrical pipe (m)

η ('eta') = the viscosity of the liquid (Pa s)

δP = the pressure difference across the ends of the pipe (Pa)

δx = the length of the pipe (m).

Verify, by putting relevant units in place of the variables in this equation and simplifying the resulting relationship, that appropriate units for J_v , the mean flow rate, are ms⁻¹.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

29 Designing experiments

Definitions

Some of the terms used when discussing scientific method may be used rather loosely in other contexts. For the purposes of this chapter, the following definitions will be assumed.

Paradigm – theoretical framework so successful and well confirmed that most research is carried out within its context and does not challenge it.

Theory – a collection of hypotheses that covers a range of natural phenomena – a ‘larger-scale’ idea than a hypothesis. Note that a theory may be ‘hypothetical’, in the sense that it is a tentative explanation.

(Scientific) Law – this concept can be summarised as an equation (law) that provides a succinct encapsulation of a system, often in the form of a mathematical relationship. The term is often used in the physical sciences (e.g. ‘Beer’s Law’, p. 338).

Hypothesis – a possible explanation for an observed phenomenon tested in a specific experiment or by a set of observations. Tends to involve a ‘small-scale’ idea.

Alternative hypothesis – a term used in two senses; more generally, as one of several possible different explanations of the phenomenon being tested in an experiment (other than the primary hypothesis), or in the field of statistics, as a binary alternative to a ‘null hypothesis’ which (generally) assumes no effect of a particular treatment (pp. 602–3).

Biology is a body of knowledge based on observation and experiment. Biologists attempt to explain life in terms of theories and hypotheses. They make predictions from these hypotheses and test them by experiment or further observations. The philosophy and sociology that underlie this process are complex topics (for more detail, see for example, Chalmers, 2013). Any brief description inevitably requires simplifications.

Following the scientific method

The systematic cycle of observation, proposing a hypothesis and testing is called ‘the scientific method’. Figure 29.1 models the scientific process you are most likely to be involved in – testing ‘small-scale’ hypotheses. These represent possible explanations for phenomena which give rise to predictions that can be tested by an experiment or a series of observations. For example, you might put forward the hypothesis that the rate of K^+ efflux from a particular cell type is dependent on the intracellular concentration of calcium ions. This might then lead to a prediction that the application of a substance known to decrease the intracellular concentration of calcium ions would reduce K^+ efflux from the cells. An experiment could be set up to test this hypothesis and the results would either confirm or not confirm (i.e. falsify or refute) the hypothesis.

If confirmed, a hypothesis is accepted and retained with greater confidence. If falsified, it is either rejected outright as false, or modified and retested. Alternatively, it might be decided that the experiment was not a valid test of the hypothesis (in the previous example, perhaps because it was later found that the applied substance could not penetrate the cell membrane to a presumed site of action).

Understanding paradigms

Nearly all scientific research deals with the testing of small-scale hypotheses. These hypotheses operate within a theoretical framework that has proven to be successful (i.e. is confirmed by many experiments and/or observations and is consistently predictive). This large-scale operating model or ‘paradigm’ is not changed readily, and, even if a single result appears that seems to challenge the conventional view, would not be overturned immediately. The conflicting result would be ‘shelved’ until an explanation was found after further investigation. In the example used above, a relevant paradigm could be the notion that life processes are ultimately chemical in nature.

Although changes in paradigms are rare, they are important, and the scientists who recognise them often become well known. For example, a ‘paradigm shift’ can be said to have occurred when Darwin’s ideas about Natural Selection replaced Special Creation as an explanation for the origin of species. Generally, however, results from hypothesis-testing tend to support and develop (‘articulate’) the paradigm, enhancing its relevance and strengthening its status. Thus, research in the area of population genetics has developed and refined Darwinism.

Developing hypotheses

Where do ideas for small-scale hypotheses come from? They arise from one or more thought processes on the part of a scientist:

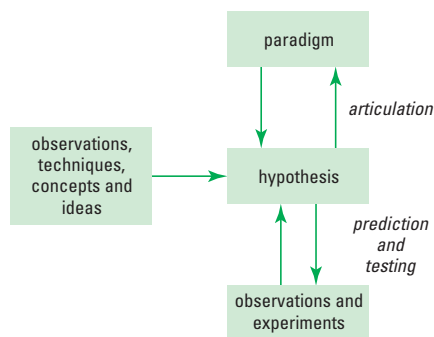


Fig. 29.1 A model of scientific method as used when testing hypotheses on a small scale. Hypotheses can arise as a result of various thought processes on the part of the scientist, and are consistent with the overlying paradigm. Each hypothesis is testable by experiment or observation, leading to its confirmation or rejection. Confirmed hypotheses act to strengthen the status of the paradigm, but rejected ones do not immediately result in the paradigm's replacement.

Deciding whether to accept or reject a hypothesis – this is sometimes clear-cut, as in some areas of genetics, where experiments can be set up to result in a binary outcome (Chapters 66–69). In many other cases, the existence of ‘biological variation’ means that statistical techniques need to be employed, with implicit acceptance of uncertainty in conclusions (Chapters 76 and 77; Box 65.2).

Definition

Mathematical model – an algebraic summary of the relationship between the variables in a system.

- analogy with another system
- recognition of a pattern
- recognition of departure from a pattern
- invention of a new analytical method
- development of a mathematical model
- intuition
- imagination.

The process of science is not an entirely objective one, as is sometimes assumed. For instance, the choice of analogy that may lead to a new hypothesis might well be subjective, depending on past knowledge or understanding. Also, science is a social activity, where researchers put forward and defend viewpoints against those who hold an opposing view; where groups may work together towards a common goal; and where effort may depend on externally dictated financial opportunities and constraints. As with any other human activity, science is bound to involve an element of subjectivity.

No hypothesis can ever be rejected with certainty. Statistics might allow us to quantify as vanishingly small the probability of an erroneous conclusion, but we are left in the position of never being 100% certain that we have rejected all relevant alternative hypotheses, nor 100% certain that our decision to reject some alternative hypotheses was correct. However, despite these problems, experimental science has yielded and continues to yield many important findings.

KEY POINT The fallibility of scientific ‘facts’ is essential to grasp. No explanation can be accepted with 100% certainty as it is always possible for a new alternative hypothesis to be generated. Our understanding of biology changes all the time as new observations and methods force old hypotheses to be retested and sometimes rejected.

Quantitative hypotheses involve a mathematical description of a system. They can be formulated concisely by mathematical models. Formulating models is often useful because it forces deeper thought about mechanisms and encourages simplification of the system. A mathematical model:

- encapsulates many observations
- is inherently testable through experiment
- identifies areas where information is lacking or uncertain
- allows you to predict the behaviour of the system.

Remember, however, that assumptions and simplifications required to create a model may result in it being unrealistic. Further, the results obtained from any model are only as good as the information put into it.

Devising experiments

An experiment is effectively an artificial situation set up by a scientist to examine a phenomenon or to test a hypothesis. In essence, the notion is to keep as many variables as constant as possible, while changing a variable

Box 29.1 How to design and perform an experiment**1. Preliminaries**

- (a) Formulate a simple hypothesis to test. It is preferable to have a clear answer to one question than to be uncertain about several questions.
- (b) Decide which dependent variable you are going to measure and how. Is it relevant to the problem? Can you measure it accurately, precisely and without bias (Chapter 27).
- (c) Think about and plan the statistical analysis of your results (Chapters 76 and 77). Will this affect your design?

2. Designing

- (a) Find out the extent of your resources.
- (b) Choose treatments and conditions that alter the minimum of confounding variables (p. 201).
- (c) Incorporate as many effective controls (p. 201) as possible.
- (d) Keep the number of replicates as high as is feasible.
- (e) Ensure that the same number of replicates is present in each treatment and control.
- (f) Use effective randomisation and blocking arrangements.

3. Planning

- (a) List all the materials you will need. Order any chemicals and make up solutions; grow, collect or

breed the experimental subjects you require; check equipment is available.

- (b) Organise space and/or time in which to do the experiment.
- (c) Account for the time taken to apply treatments and record results. Make out a timesheet if things will be hectic.

4. Carrying out the experiment

- (a) Record the results and make careful notes of everything you do (see Chapter 31). Make additional observations to those planned if interesting things happen.
- (b) Repeat experiment if time and resources allow.

5. Analysing

- (a) Graph data as soon as possible (during the experiment if you can). This will allow you to visualise what has happened and make adjustments to the design (e.g. timing of measurements).
- (b) Carry out the planned statistical analysis.
- (c) Jot down conclusions and new hypotheses arising from the experiment.

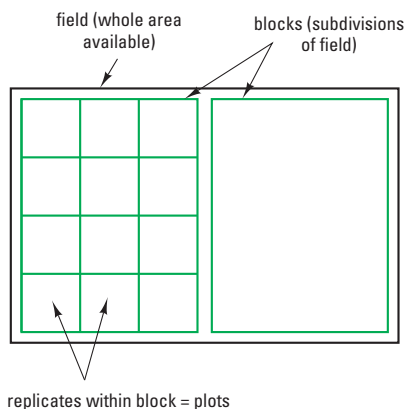


Fig. 29.2 Terminology and physical arrangements of elements in an experiment. Each block should contain the complete range of treatments (treatments may be replicated more than once in each block).

of interest. Box 29.1 outlines the important stages in designing and performing an experiment. In many cases, the aim is to provide evidence for causality (a ‘cause and effect’ relationship). If x causes y , we expect, repeatedly, to find that a change in x results in a change in y . Hence, the ideal experiment of this kind involves measurement of y , the dependent (measured) variable, at one or more values of x , the independent variable, and subsequent demonstration of some relationship between them. Experiments therefore involve comparisons of the results of treatments – changes in the independent variable as applied to an experimental subject. A ‘control’ is another treatment where either no change is made, or a potentially confounding variable (p. 201) is adjusted so that its effects, if any, can be taken into account. There are often many potential controls for any experiment.

Subjects given the same treatment are known as replicates (they also may be called plots). A block is a grouping of replicates or plots. The blocks are contained in a field, i.e. the whole area (or time) available for the experiment (Fig. 29.2). These terms originated from the statistical analysis of agricultural experiments, but they are now used for all areas of biology.

Controlling variables in experiments

For some experiments, the results may be clear-cut, for example where removal of part of a gene affects its expression in an ‘on or off’ manner.

Example Suppose you wish to investigate the effect of a metal ion on the growth of a bacterial culture. If you add the metal as a salt to the culture and then measure the growth, you will immediately introduce at least two confounding variables, compared with a treatment where no salt is added. Firstly, you will introduce an anion that may also affect growth in its own right, or in combination with the metal ion; secondly, you will alter the osmotic potential of the medium (see pp. 158–9). Both of these effects could be tested using appropriate controls. For instance, you might test for osmotic effects by comparing results with the metal salt versus results using an inert osmotically active compound instead (Fig. 29.3).

Definition

Control – a treatment carried out to investigate the possibility that factors other than the one(s) being studied will influence the results obtained.

Accounting for edge effects – these may exist in experiments where treatments are laid out in a spatial array. These effects can be reduced by incorporating a ‘buffer zone’ of untreated subjects around the experiment proper.

However, in many other types of investigation, the effects of treatments may occur alongside a background of considerable variability in responses. As a result, interpretation of such experiments is seldom straightforward, although careful experimental design and data analysis can assist in separating out different forms of variability from ‘real’ responses (Figs 29.3 and 29.4).

Confounding variables (systematic variation)

These increase or decrease systematically as the independent variable increases or decreases. Their effects are known as systematic variation. For example, if you wish to determine the effect of a drug on a cellular reaction, it may only be available as an acidic compound, and by adding it to the relevant medium you might also adjust the pH at the same time, potentially affecting the results due to other effects on cellular processes. pH is thus a confounding variable in that example. This form of variation can be disentangled from that caused directly by treatments by incorporating appropriate controls in the experiment. The results from a control may therefore allow you to reject an alternative hypothesis (that the relevant adjustment was the real reason for the response, and not the treatment). In the example above this might be by using buffers (p. 167) to test the effect of pH directly.

The consequence of systematic variation due to confounding variables is that you can never be certain that the treatment, and the treatment alone, has caused an observed result. By careful design, you can, however, minimise the uncertainty involved in your conclusion. Methods available include:

- **ensuring, through experimental design, that the independent variable is the only major factor that changes in any treatment**
- **incorporating appropriate controls** to show that potential confounding variables have little or no effect
- **selecting experimental subjects randomly** to cancel out systematic variation arising from biased selection
- **matching or pairing individuals among treatments** so that differences in response due to their initial status are eliminated
- **arranging subjects and treatments randomly** so that responses to systematic differences in conditions do not influence the results
- **ensuring that experimental conditions are uniform** so that responses to systematic differences in conditions are minimised.

Nuisance variables (random variation)

These are uncontrolled variables that cause differences in the value of y independently of the value of x , resulting in random variation, in contrast to the systematic variation caused by confounding variables. Figure 29.4 illustrates the potential effects of nuisance variables on the scatter of data around the mean value. Experimental biology is characterised by the high number of nuisance variables that are present and their relatively great influence on results: biological data tend to have large errors. To reduce and assess the consequences of nuisance variables:

- **incorporate replicates to allow random variation to be quantified**
- **choose subjects that are as similar as possible**
- **control random fluctuations in environmental conditions**
- **increase the number of replicates used.**

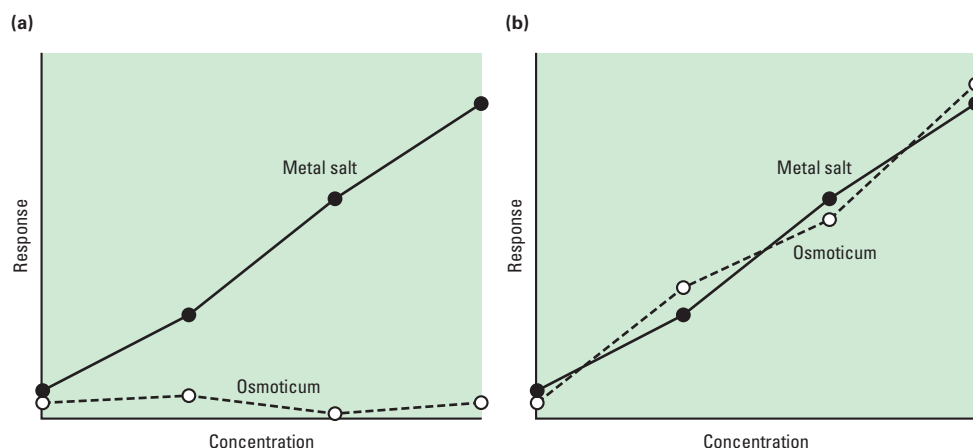


Fig. 29.3 Alternative potential results from an experiment testing the effects of a metal salt on a physiological response. In each case, a parallel treatment of inert osmoticum only (at equivalent solute potential, p. 155) has been added as a control to test for osmotic effects of the salt. In scenario (a) the osmotic control results in little or no response, indicating little or no osmotic effect on the response; in (b), however, the osmotic control results in a similar response to the metal salt, indicating that the apparent effects of the salt might in fact be due to changes in the solute potential of the medium (further experiments might test this hypothesis directly) and therefore that osmotic potential might be a confounding variable.

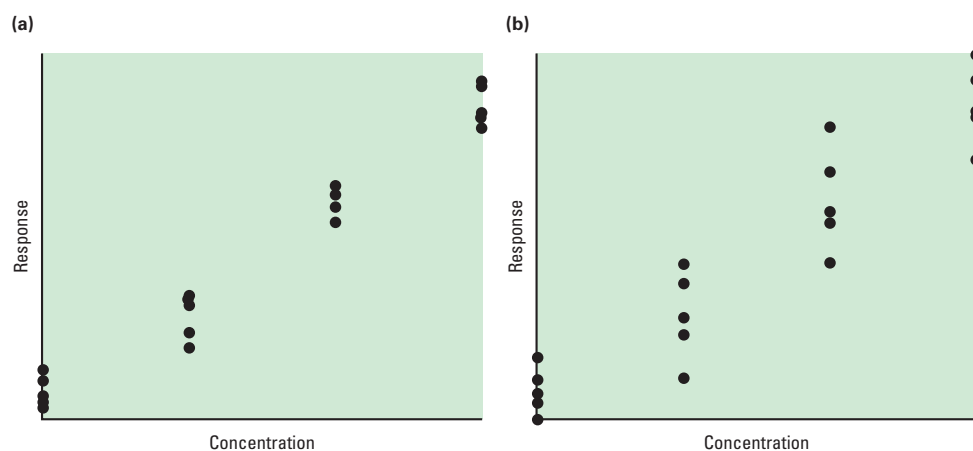


Fig. 29.4 Alternative potential results from the experiment shown in Fig. 29.3 illustrating the effects of a metal salt on a physiological response. Here, the individual data values are shown, rather than the mean values alone. Both graphs show the same mean response at the different salt concentrations, but in (a) the combined effects of nuisance variables (random variation), as shown by the scatter of data values, is relatively low, whereas in (b) it is relatively high.

The effect of nuisance variables is to reduce the confidence that can be given to conclusions about your data. The best way to account for them is via appropriate statistical tests (Chapter 77) that will allow you to assign a probability value to your conclusions.

Dealing with constraints on experimental design

At an early stage, you should find out how resources may constrain the design. For example, limits may be set by availability of subjects, cost of treatment, availability of a chemical or bench space. Logistics may be a factor (for example, the time required to record or analyse data).

Evaluating design constraints – a good way to do this is by processing an individual subject through the experimental procedures – this type of ‘preliminary run’ can help to identify potential difficulties.

Deciding the number of replicates in each treatment – try to:

- maximise the number of replicates in each treatment
- make the number of replicates the same for each treatment.

Understanding pseudo-replication – this term applies to a situation where replicates are used, but they are not fully independent of each other. An example might occur when testing the effect of a chemical on liver function, if several samples from one animal's organ were treated as independent replicates, rather than a sample from the livers of each of several animals. In practice, an approach which examined both intra- and inter-animal variability might be the best way to proceed, using several samples from each of several animals, then analysing the results using ANOVA (Chapter 77).

Example If you know that light varies in a graded fashion within a cabinet used to culture cyanobacteria, you might arrange blocks to be long thin rectangles at right angles to the gradient to ensure conditions within the block are as even as possible.

Your equipment or facilities may affect design because you cannot regulate conditions as well as you might desire. For example, you may be unable to ensure that temperature and lighting are equal over an experiment laid out in a glasshouse or you may have to accept a great deal of initial variability in your subjects. These problems are especially acute for experiments carried out in the field or subjects collected from the wild.

Using replicates

Replicate results show how variable the response is within treatments. They allow you to compare the differences *between* treatments in the context of the variability *within* treatments – you can do this via statistical tests such as analysis of variance (Chapter 77). Larger sample sizes tend to increase the precision of estimates of parameters and increase the chances of showing a significant difference between treatments if one exists. For statistical reasons (weighting, ease of calculation, fitting data to certain tests), it is often best to keep the number of replicates even among treatments. Remember that the degree of independence of replicates is important: subsamples cannot act as replicate samples – they tell you about variability in the measurement method but not in the quantity being measured.

If the total number of replicates available for an experiment is limited by resources, you may need to compromise between the number of treatments and the number of replicates per treatment. Statistics can help here, for it is possible to work out the minimum number of replicates you would need to show a certain difference between pairs of means (say 10%) at a specified level of significance (say $P = 0.05$). For this, you need to obtain a prior estimate of variability within treatments perhaps from an initial trial run (see Sokal and Rohlf, 2012).

Randomising treatments

This is important to minimise the possibility of erroneous results arising from unbalanced selection of individuals for different treatments. The two aspects of randomisation you must consider are:

- **positioning of treatments within experimental blocks**
- **allocation of treatments to the experimental subjects.**

For relatively simple experiments, you can adopt a completely randomised design; here, the position and treatment assigned to any subject is defined randomly. You can draw lots, use a calculator or Web-based random number generator, or use the random number tables that can be found in most books of statistical tables (see Box 29.2).

A completely randomised layout has the advantage of simplicity but cannot show how confounding variables alter in space or time. This information can be obtained if you use a blocked design in which the degree of randomisation is restricted. Here, the experimental space or time is divided into blocks, each of which accommodates the complete set of treatments (Fig. 29.2). When analysed appropriately, the results for the blocks can be compared to test for differences in the confounding variables across the field and these effects can be separated out from the effects of the treatments. The size and shape (or timing) of the block you choose is important: besides being able to accommodate the number of replicates desired, the suspected confounding variable should be relatively uniform within the block.

Box 29.2 How to use random number tables to assign subjects to positions and treatments

This is one method of many that could be used. It requires two sets of n random numbers – where n is the total number of subjects used.

1. **Number the subjects in any arbitrary order but in** such a way that you know which is which (i.e. mark or tag them).
2. **Decide how treatments will be assigned**, e.g. first five subjects selected, treatment A; second five – treatment B, etc.
3. **Use the first set of random numbers in the sequence obtained to identify subjects and allocate them to treatment groups** in order of selection as decided in (2).
4. **Map the positions for subjects in the block or field. Assign numbers to these positions using the second set of random numbers**, working through the positions in some arbitrary order, e.g. top left to bottom right.
5. **Match the original numbers given to subjects with the position numbers.**

To obtain a sequence of random numbers:

1. **Decide on the range of random numbers you need.**
2. **Decide how you wish to sample the random number tables** (e.g. row by row and top to bottom) and your starting point.

3. **Moving in the selected manner, read the sequence of numbers until you come to a group that fits your needs** (e.g. in the sequence 978186, 18 represents a number between 1 and 20). Write this down and continue sampling until you get a new number. If a number is repeated, ignore it. Small numbers need to have the appropriate number of zeros preceding (e.g. 5 = 05 for a range in the tens, 21 = 021 for a range in the hundreds).
4. **When you come to the last number required, you do not need to sample any more:** simply write it down.

Example: You find the following random number sequence in a table and wish to select numbers between 1 and 10 from it.

9059146823	4862925166	1063260345
1277423810	9948040676	6430247598
8357945137	2490145183	5946242208
6588812379	2325701558	3260726568

Working left to right and top to bottom, the order of numbers found is 5, 10, 3, 9, 4, 6, 2, 1, 8, 7 as indicated by blue type. If the table is sampled by working row by row right to left from bottom to top, the order is 6, 10, 7, 2, 9, 3, 4, 8, 1, 5.

3 × 3			4 × 4			
A	C	B	A	C	B	D
B	A	C	D	B	C	A
C	B	A	C	D	A	B
			B	A	D	C

Fig. 29.5 Examples of Latin square arrangements for 3 and 4 treatments. Letters indicate treatments; the number of possible arrangements for each size of square increases greatly as the size increases.

A Latin square is a method of placing treatments so that they appear in a balanced fashion within a square block or field. Treatments appear once in each column and row (see Fig. 29.5), so the effects of confounding variables can be ‘cancelled out’ in two directions at right angles to each other. This is effective if there is a smooth gradient in some confounding variable over the field. It is less useful if the variable has a patchy distribution, where a randomised block design might be better.

Latin square designs are useful in serial experiments where different treatments are given to the same subjects in a sequence (for example, Fig. 29.6). A disadvantage of Latin squares is the fact that the number of plots is equal to the number of replicates, so increases in the number of replicates can only be made by the use of further Latin squares.

Pairing and matching subjects

The paired comparison is used to reduce systematic variation when there are two treatments. Examples of its use are:

- **‘Before and after’ comparison.** Here, the pairing removes variability arising from the initial state of the subjects, for example, weight gain of mice on a diet, where the weight gain may depend on the initial weight.

		Animal				
		1	2	3	4	5
Month	1	A	C	D	B	E
	2	D	B	E	A	C
	3	E	D	B	C	A
	4	C	E	A	D	B
	5	B	A	C	E	D

Fig. 29.6 Example of how to use a Latin square design to arrange sequential treatments. The experimenter wishes to test the effect of drugs A–E on weight gain, but has only five animals available. Each animal is fed on the control diet for the first 3 weeks of each month, then on the control diet plus drug for the last week. Weights are taken at the start and finish of each treatment. Each animal receives all treatments.

Definition

Interaction – where the effect of treatments given together is greater or less than the sum of their individual effects.

		Factor B	
		–	+
Factor A	–	0	b
	+	a	$a + b + c$

Fig. 29.7 Design of a simple multifactorial experiment. Factors A and B have effects a and b when applied alone. When both are applied together, the effect is denoted by $a + b + c$.

- If $c = 0$, there is no interaction (e.g. $2 + 2 + c = 4$).
- If c is positive, there is a positive interaction (synergism) between A and B (e.g. $2 + 2 + c = 5$).
- If c is negative, there is a negative interaction (antagonism) between A and B (e.g. $2 + 2 + c = 3$).

- **Application of a treatment and control to parts of the same subject or to closely related subjects.** This allows comparison without complications arising from different origin of subjects, for example, drug or placebo given to sibling rats, virus-containing or control solution swabbed on left or right halves of a leaf.
- **Application of treatment and control under shared conditions.** This allows comparison without complications arising from different environments of subjects, for example, rats in a cage, plants in a pot.

Matched samples represent a restriction on randomisation where you make a balanced selection of subjects for treatments on the basis of some attribute or attributes that may influence results, for example, age, sex, prior history. The effect of matching should be to ‘cancel out’ the unwanted source(s) of variation. Disadvantages include the subjective element in choice of character(s) to be balanced, inexact matching of quantitative characteristics, the time matching takes and possible wastage of unmatched subjects.

When analysed statistically, both paired comparisons and matched samples can show up differences between treatments that might otherwise be rejected on the basis of a fully randomised design, but note that the statistical analysis may be different.

KEY POINT Sometimes you will need to consider external influences on experimental design. These might include professional codes of conduct and a number of matters relating to ethics, such as animal welfare, safeguarding ecological sites and obtaining informed consent from any human subjects (Chapter 30).

Carrying out multi-factorial experiments

The simplest experiments are those in which one treatment (factor) is applied at a time to the subjects. This approach is likely to give clear-cut answers, but it could be criticised for lacking realism. In particular, it cannot take account of interactions among two or more conditions that are likely to occur in real life. A multi-factorial experiment (Fig. 29.7) is an attempt to do this; the interactions among treatments can be analysed by specialised statistics.

Multi-factorial experiments are economical on resources because of ‘hidden replication’. This arises when two or more treatments are given to a subject because the result acts statistically as a replicate for each treatment. Choice of relevant treatments to combine is important in multi-factorial experiments; for instance, an interaction may be present at certain concentrations of a chemical but not at others (perhaps because the response is saturated). It is also important that the measurement scale for the response is consistent, otherwise spurious interactions may occur. Beware when planning a multi-factorial experiment that the numbers of replicates do not get out of hand: you may have to restrict the treatments to ‘plus’ or ‘minus’ the factor of interest (as in Fig. 29.7).

Reporting correctly – it is good practice to report how many times your experiments were repeated (in Materials and Methods); in the Results section, you should add a statement saying that the illustrated experiment is representative.

Repeating experiments

Even if your experiment is well designed and analysed, you should recognise that only limited conclusions can be made. Firstly, what you can say is valid for a particular place and time, with a particular investigator, experimental subject and method of applying treatments. Secondly, if your results were significant at the 5% level of probability (p. 603), there is still an approximately 1-in-20 chance that the results did arise by chance. To guard against these possibilities, it is important that experiments are repeated. Ideally, this would be done by an independent scientist with independent materials. However, it makes sense to repeat work yourself so that you can have full confidence in your conclusions. Many research scientists recommend that experiments are done three times in total, but this may not be appropriate in undergraduate work.

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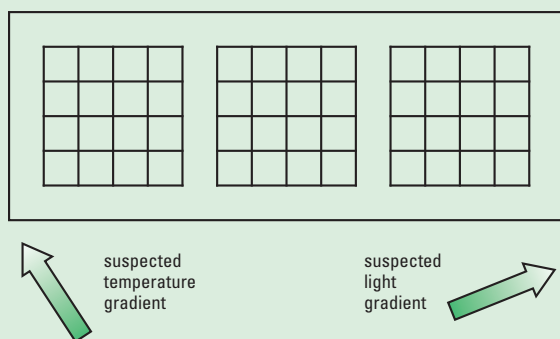
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STUDY EXERCISES

29.1 Create a Latin square design. Treatments W, X, Y and Z are to be applied to potted plants in a glasshouse where the researcher suspects there may be a slight gradation in temperature and light over an oblong bench that can hold a total of 48 pots. You decide to use an experimental design consisting of three 4×4 blocks of plants, each arranged in a different Latin square design (see below). Assign treatments to the locations in the diagram below. Explain why this design will help to eliminate the effects of the confounding variables.



29.2 Generate random numbers. Produce a list of 20 random whole numbers between 1 and 5. This can be carried out using a spreadsheet. If using Microsoft *Excel*, investigate the `RAND()` and `INT` functions. The `RAND()` function produces a random number between 0 and 1, so you will need to multiply by a constant factor to scale your final output appropriately. Copy your test

formula(e) to several cells to test empirically whether it works.

29.3 Investigate possible interactions. Treatments A, B and C involve tests of three different nutrients on proliferation of cells in a culture of carrot cells. Analyse the results in the table below to determine whether any interactions have occurred between the four possible combinations of treatments. No complex statistical analysis is required, simply a calculation of mean experimental effect (treatment minus controls) in each combined treatment and a comparison with the data for the relevant treatments on their own (in other words, assume that observed differences reflect true underlying differences). Classify the results as 'no interaction', 'antagonism' or 'synergism'

Results of cell proliferation experiment

Treatment	Replicate (growth of callus in g)				
	1	2	3	4	5
Control	4.8	5.2	5.2	4.8	5.0
A	6.5	7.3	7.0	7.1	7.1
B	7.7	8.3	8.5	7.5	8.0
C	10.7	10.0	9.9	9.8	9.6
A + B	6.6	7.2	6.8	7.2	7.2
A + C	11.9	11.7	12.0	12.0	12.4
B + C	17.3	16.8	17.1	16.6	17.2
A + B + C	12.8	12.9	13.1	13.3	12.9

Answers to these study exercises are available at go.pearson.com/uk/he/resources

30 Understanding bioethics

Definitions

Values – beliefs held by individuals and groups; for example, religious, cultural or political values.

Morals – precepts and principles aimed at distinguishing right from wrong; for example, is it right to test pharmaceutical products on animals?

Ethics (moral philosophy) – rules that define appropriate behaviour, based on moral principles; for example, research ethics and bioethics (applied ethics).

Note that the practical distinction between morals and ethics sometimes is less clear-cut than defined above, and the terms are occasionally used interchangeably.

Recognising the difference between morals and ethics – consider a biomedical scientist who believes that murder is fundamentally wrong (immoral). However, the scientist's research into a specific disease requires experiments that will lead to the death of some animals, to be carried out under a code of research ethics that specifies that the animals should not endure unnecessary pain or suffering. What should the scientist do? You will find that many aspects of bioethics are best considered through specific examples of dilemmas such as this.

Understanding the terminology of issues and dilemmas – these topics are sometimes referred to as ethical, legal and social issues (ELSI) or aspects (ELSA).

Definition

Xenotransplantation – surgical transfer of tissues/organs from one species to another, typically discussed in terms of animal-to-human xenotransplantation.

The philosophical study of ethical issues arising from recent advances in biology and medicine is termed 'bioethics'; its scope also extends to cover environmental and global issues. It aims to provide a framework for making decisions, based on specific moral principles, taking into account the reasons underlying different choices and the consequences of specific decisions. Particular groups of individuals will often adopt a set of ethical rules and standards as part of their professional code of conduct, for example, medical ethics.

This chapter provides you with a basic framework that will help you understand the various ethical theories and principles, so that you can consider some of the issues in contemporary biology. Without an ethical framework, individuals can only make subjective 'value judgements', based solely on personal opinions and viewpoints.

Bioscience degree programmes place increasing emphasis on the ethical and social impacts of contemporary scientific advances and on the need for scientists engaged in potentially controversial work to communicate their ideas and decision-making to the general public. Bioscience research raises many moral, ethical and legal dilemmas, requiring difficult choices to be made, for example, in relation to animal testing of medical products, and students are likely to be asked to reflect on bioethical questions and dilemmas, for example, in group debates. In discussing such issues with others, you will gain experience that will have broader value, in terms of understanding some of the issues linked to the public understanding of science, and of how these issues can be addressed through communication with the public. You will find links and resources addressing this broader topic on the websites of a number of professional scientific organisations such as the Royal Society of London, and the Wellcome Trust in the UK, or the Coalition on the Public Understanding of Science (COPUS) in the USA.

KEY POINT In contrast to numerical calculations (Chapter 75) there is rarely a single 'right' or 'wrong' answer to bioethical issues. Consequently, it is important to use ethical principles and systems to consider each issue in a logical manner. Your aim should be to develop your skills in critical thinking and reasoning (p. 210), and in explaining how you have arrived at a particular decision on a bioethical issue, with sound evidence and a reasoned argument (p. 210) to support the position that you have taken.

Bioethics can be subdivided into three main areas:

1. **environmental ethics** – for example, the use of genetically modified organisms (GMOs), the exploitation/destruction of native habitats
2. **animal ethics** – examples include factory farming, the use of transgenic animals and xenotransplantation
3. **human ethics** – dealing with medical and social issues, such as human cloning, embryo and stem cell research, CRISPR-Cas9 'gene editing' (p. 513), genetic testing and the storage of DNA profiles on databases.

Understanding ethical theories

A basic understanding of the various theories of ethics may help you formulate your ideas in relation to specific questions and issues. The major theories include:

- **Utilitarianism** – the notion that it is right (ethical) to choose the action that produces the greatest good (happiness) for the greatest number. For example, the use of atomic bombs against civilians in the Japanese cities of Hiroshima and Nagasaki in World War II was justified in terms of its utilitarian value, in terms of ending the war and preventing Japan's development and use of similar weapons, with potentially greater loss of life on both sides.
- **Deontology** – the theory that states that a particular action is either intrinsically good (right) or bad (wrong), and that such principles are absolute. According to deontological theory, decisions must be based on the actions themselves, rather than on their overall consequences. Such an approach is enshrined in the Judaeo-Christian religious value system (for example, in the ten commandments of the Old Testament). While this approach appears simple to apply, for example, in upholding the principle that 'it is always wrong to lie', it does not allow for particular circumstances, for example, is it wrong to lie if, in doing so, you could prevent a murder? Perhaps in this example of an ethical dilemma, most people would agree that the greater 'good' of preventing a murder would outweigh the lesser 'good' of not lying, though this then moves towards utilitarianism.
- **Consequentialism** – where decisions are made and choices are selected by considering their likely outcomes and consequences, rather than from any intrinsic viewpoint. From a consequentialist viewpoint, a lie would be the right course of action to take if it prevented a murder.
- **Virtue theory** – arising from the philosophy of Plato and Aristotle, this enshrines the notion that making decisions according to established virtues, such as honesty, wisdom, justice and altruism will lead to ethically valid choices. As with consequentialism and utilitarianism, virtue theory is distinct from deontology, since it is not based on rigid absolutes.
- **Objectivism** – the theory that what is right and wrong is intrinsic and applies equally to all people, places and times. The alternative is that morality is subjective, being entirely dependent upon the views of the individual (**subjectivism**).

Understanding consequentialism – this theory is probably best summed up by the phrase 'the end justifies the means'.

Definition

Altruism – the virtue of selfless concern for the welfare of others.

Reflecting on ethical principles

The following provides a framework of fundamental principles for considering particular topics and questions from different perspectives:

- **Beneficence** – the obligation to do good. For example, if it is possible to prevent suffering by a particular course of action, then it should be carried out. However, dilemmas arise in particular circumstances when conflicts arise; for example, is it acceptable to cause suffering to animals used in medical research that aims to prevent suffering to humans in the future?

Examples Current bioethical issues and dilemmas:

- Whether to locate wind farms in the Scottish highlands. (Weigh up the benefits of 'green energy' against those of damage to environmentally sensitive areas.)
- Whether vivisection (using dissection procedures on living animals) can be justified if it may lead to medical advances that reduce human suffering.
- Whether the potential advantages of using GMOs outweigh the possible disadvantages (e.g. the use of 'golden rice', which has been genetically modified to contain genes for vitamin A synthesis, as a means of tackling vitamin A deficiency in some developing countries, versus widespread malnutrition in the population).

Definition

Informed consent – the agreement provided by a participant in a medical or research study, following full disclosure of the purpose of the study and the possible risks involved (see p. 211 for more details).

Example An insidious scientific fraud: Andrew Wakefield, a former doctor who was struck off the UK medical register for promoting 'misleading, dishonest and irresponsible' claims that there was a link between measles-mumps-rubella (MMR) vaccination and autism, a conclusion which was shown to be based on seriously flawed research. For more examples, see journalist Ben Goldacre's 'Bad Science' website at: <https://www.badscience.net/>.

- **Non-maleficence** – the duty to do no harm. This is enshrined within the Hippocratic oath of medical practitioners, but is not without difficulty in its practical application in specific circumstances. For example, should a medical practitioner continue to maintain someone who is on a life-support machine and who has no reasonable expectation of recovery, if this is causing anguish to the patient's family, and if the practitioner knows that another person is awaiting life support? Here, the practitioner has to weigh up the different aspects of the case, and make a balanced judgement.
- **Justice** – the obligation to treat all people fairly and impartially; for example, lack of discrimination between people on the grounds of race, colour, sex, language, religion, age or other status. This principle is also enshrined in Article 2 of the Universal Declaration of Human Rights (United Nations, 1948).
- **Autonomy** – the duty to allow an individual to make their own choices, without constraints. This principle underlies the notion of 'informed consent' in medical and scientific research.
- **Respect** – the need to show due regard for others; for example, the need to take into account the rights and beliefs of all people equally. This is also an intrinsic component of the UN Universal Declaration of Human Rights. It is also an essential component of the principle of confidentiality of personal information about participants in medical and scientific research.
- **Rationality** – the notion that a particular action of choice should be based on reason and logic. Many scientists would argue that the scientific method itself (pp. 198–9) is a good example of the principle of rationality in action.
- **Honesty** – the obligation to be truthful. This principle also underpins 'informed consent', since individuals cannot make informed choices if they have not been provided with information that is true and complete. It is also a fundamental aspect of scientific research ethics – scientists have a duty to present their results as they truly are, without falsification or bias. When this principle is breached, the misconduct is often termed 'scientific fraud'.
- **Precautionary principle** – the notion that it is better not to carry out an action if there is any risk of harm. For example, many people would apply the precautionary principle in deciding that the risks of building nuclear power stations outweigh their potential benefits. It is enshrined in principle 15 of the Rio Declaration (United Nations, 1992) as follows:

to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation.

It is also an aspect of sustainable approaches to global issues, such as global warming. While the precautionary principle is widely applied to environmental ethics, it is also relevant to other areas. For example, in medical ethics, should a doctor recommend a novel vaccine to counter COVID-19 when only limited information is available about possible long-term side effects?

It is clear from the above listing, that several different principles may apply to a particular bioethical issue, and it will be up to individuals to weigh up the relative merits and disadvantages of particular choices and options in terms of the different outcomes against these principles when faced with an 'ethical dilemma'. Box 30.1 outlines a practical and sequential approach to making ethical decisions.

Seeking ethical approval – note that many universities have separate committees for human ethics and animal ethics – you should consult your supervisor for specific advice well in advance of your planned start date, since your work cannot proceed until it has ethical approval, and this will need to fit in with the timelines of the relevant ethics committee.

Researching with human subjects

As explained above, a number of ethical principles apply to the conduct of any research involving human subjects. Such principles apply in addition to those of health and safety (Chapter 20). If you carry out *any* research involving human participants as part of your degree programme, including surveys (p. 226), you will need to obtain ethical approval before you begin any practical work. Usually, this will involve submission of a request for ethical approval of your proposed project to your institution's ethics committee.

KEY POINT For the purposes of most undergraduate research proposals, while a student may be the investigator, the responsibility for following appropriate ethical approval procedures will remain with the supervisor.

Outlined below are the main aspects that you should consider in all research involving human participants, irrespective of whether the research

Box 30.1 How to make ethical decisions

The following text is based on the framework of Shamoo and Resnik (2009):

- 1. Define the issue or dilemma.** Typically this will be in the form of a question that requires a choice to be made, for example: 'Should the UK allow euthanasia?' The problem/question will often be given to you as part of a coursework assignment.
- 2. Research the literature and gather together relevant information.** This may include (i) finding scholarly papers that consider the ethics of the topic, e.g. medical ethicists who have considered euthanasia from a particular standpoint, (ii) seeking other perspectives, e.g. those of organisations in favour of and opposed to euthanasia.
- 3. Work out what options are available.** For example, you might consider under what circumstances euthanasia might be permitted, and how this would be regulated, rather than restricting yourself to a 'yes' or 'no' answer.
- 4. Consider the options in relation to ethical theories and principles.** For example, what outcomes would fit with deontological theory, or utilitarianism? How do the principles of non-maleficence, autonomy and respect for the individual apply in relation to euthanasia?
- 5. Consider whether anything else should be taken into account.** This step provides an opportunity for a 'second check' on whether you have considered all aspects of the topic, e.g. in relation to euthanasia, have you considered whether the views of relatives would carry any weight? Or whether your decision would apply to an individual suffering from a psychological disorder?
- 6. Make your decision** – choose a particular response to the question posed at the outset. In relation to euthanasia, this might be an unqualified 'yes' or 'no', or might take the form of a conditional response, e.g. 'only in the following circumstances ...'
- 7. Explain your choice** – use the evidence gained in steps 2–5 to justify the decision made at step 6. In an assessed coursework assignment, this component of your written text will probably represent the greatest proportion of the marks available (always remember that it is not about reaching the 'right' or 'wrong' answer, but about reaching a logical and reasoned decision, based on interpretation of the issue in relation to sound ethical principles and reasoned discussion, supported by published sources).

Obtaining ethical approval for a research study – many universities have a streamlined procedure for the consideration and approval of projects considered as ‘low risk’; discuss with your supervisor to see whether your study is likely to fall within this category, or whether it requires ‘full’ ethical approval.

Definition

Plain English – a clear, succinct style that aims to be direct and natural, avoiding jargon, difficult language or complex structure. This will allow the meaning of the text to be understood by people from all walks of life.

UK data protection legislation – the Data Protection Act 2018 covers the procedures that must be followed in relation to storage, use and disposal of personal data, particularly sensitive information, such as racial origins, religious beliefs and sexuality.

is ‘invasive’ (for example, collecting mouth swabs from participants to study a particular DNA sequence) or ‘non-invasive’ (for example, questioning participants about their drinking habits as part of a survey on the effects of alcohol consumption on study patterns and exam performance).

Providing information

All participants should be informed in writing and through oral explanation about the main aspects of the research study, typically through the use of a Participant Information Sheet that should include the following aspects:

- **an invitation to participate in the study**, with an explanation that participation is entirely voluntary and that the person is free to withdraw from the study at any stage
- **a succinct outline of the purpose of the study, and its likely outcomes**
- **a summary of what the participant will be expected to do, and the timelines involved**
- **a statement of any possible benefits or risks involved in participation in the study**
- **an assurance of confidentiality and anonymity in use of data from the study**, including details of how long the data will be retained within the institution
- **information about the funding source(s)**
- **names and contact details of researchers/supervisors.**

Many institutions provide templates for such information sheets, which help students to address all of the key aspects of the proposed study. The Participant Information Sheet should be written in plain English so that it can be readily understood. Researchers should also answer any additional questions that participants might have, based on their reading of the information sheet.

Seeking informed consent

After reading the Participant Information Sheet, subjects are then requested to complete and sign an Informed Consent Form. As with the Participant Information Sheet, most institutions will provide a suitable template for student use.

Assuring confidentiality

All participants must be assured that their personal details will be protected through processes that ensure anonymity. This means that all data recording and storage must be de-identified, for example, through the use of randomly assigned codes to individuals, rather than using names. In addition to ethical considerations relating to storage and use of personal information, you must also comply with any legislation relating to data storage and protection, in terms of how it is held and used, the time-limit for maintaining the information and how it will be destroyed at the end of this period.

Box 30.2 provides practical advice that should help ensure that your research study is conducted in accordance with ethical principles.

Taking account of animal welfare legislation in the UK – the Animal Welfare Act 2006 is designed to prevent animal cruelty. Before any research activities are carried out, they must be approved through a licence issued by the Home Office, under the Animals (Scientific Procedures) Act 1976.

Using animals in teaching and research

In addition to complying with any legislative requirements, such as laws relating to animal welfare, all teaching activities and research studies involving vertebrates and cephalopods (which have been recognised as being capable of feeling pain, suffering distress and suffering lasting harm) must follow the ethical guidelines of the institution, which will include seeking ethical approval in advance of any procedure involving live animals. Such approval must also be sought in relation to killing of animals for teaching or research purposes.

If you are involved in animal experimentation as part of a research project (Chapter 32), you must take care to follow all conditions of the animal licence obtained by your institution – consult your supervisor if you are unsure of any aspect of the regulations.

Box 30.2 How to adopt a step-wise approach to conducting ethical research

The following text is based on McMillan and Weyers (2013):

- 1. Research your institution's guidelines and procedures for research ethics.** Typically, such material, including general guidelines, templates and forms, will be available through the university website. For discipline-specific information, you may need to consult your departmental website, or seek advice from your supervisor. Investigating these procedures and guidelines at the outset will help you understand (i) the aspects of your own study that require ethical approval and (ii) how these might be addressed.
- 2. Consider the ethical dimensions of your project.** Are there any examples of unethical behaviour in the scientific literature or popular media that might impact on the design or implementation of your study? By taking a 'Devil's advocate' stance and thinking about your study from different perspectives, you should be able to consider any potential ethical flaws in your proposed experimental design (see also Box 30.1).
- 3. Discuss your proposed research with other students and staff.** Such debate will help raise your awareness of any issues that might impact on your proposed study, and will help you 'defend' your proposal in your application for ethical approval and also in your final report.
- 4. Modify the proposed study to avoid ethical problems.** If steps 2 or 3 raise any ethical issues, then it is best to consider how best to modify your experimental design to address these. For example, if self-analysis or peer-group discussion has identified that the research might pose particular risks to specific groups of participants, then you might wish to consider excluding such groups. However, you would also need to consider the implication of such a move to your study outcomes.
- 5. Complete all necessary paperwork in advance of your study.** Remember that no experimental work can begin until you have formal written approval from the relevant ethics committee.
- 6. Carry out all practical procedures and data collection/storage as required by guidelines and legislation.** Remember that this is an important aspect of your personal development and a key aspect of professional practice that will apply when you graduate. All research scientists are bound by the same requirements to conduct their research within an ethical framework.

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STUDY EXERCISES

30.1 Test your understanding of the terminology and theories of ethics. Distinguish between the following pairs of terms:

- (a) morals and ethics
- (b) value judgements and ethical decisions
- (c) utilitarianism and deontology
- (d) consequentialism and virtue theory
- (e) objectivism and subjectivism.

30.2 Test your understanding of ethical principles. Explain each of the following terms, in the context of ethics:

- (a) beneficence
- (b) non-maleficence
- (c) justice
- (d) autonomy
- (e) precautionary principle.

30.3 Locate Web-based information on bioethics posted by relevant organisations and learned societies.

This could be important if you are researching a topic with an ethical dimension, e.g. an essay on the pros and cons of genetic modification of farm animals, or if you are considering a research project involving human or animal subjects.

30.4 'Brainstorm' the ethical issues within a research project or topic.

Make a 'spider diagram' (p. 69) or 'Mind Map' (p. 27) with your title or topic at the centre. Then, use the ethical framework described in this chapter to consider the various aspects of the title/topic, and the choices, decisions and actions required. You could carry out this exercise on an individual basis, or as a group exercise (share your spider diagrams or mind maps with other students, and discuss your views and those of others within the group).

Answers to these study exercises are available at go.pearson.com/uk/he/resources

31 Recording data and images

In contrast to the early years of your degree studies, where your practical work and reporting will often be guided by detailed schedules (p. 216), in subsequent advanced activities and projects, you will need to master the important skills of recording and managing data. This is important for the following reasons:

- **an accurate record helps when using information later**, perhaps for exam purposes or when writing a report
- **using appropriate recording procedures enables you to practise important skills** such as scientific writing, drawing diagrams, photography and imaging, preparing graphs and tables and interpreting results
- **analysing and writing up your data as you go along prevents backlogs** and helps with time management (Chapter 2)
- **you can show your work to a future employer** to prove you have developed the skills necessary for recording and reporting your work; in industry, this is vital so that others in your team can interpret and use your results.

Learning from your results – effective recording and analysis of your data may suggest options for subsequent experiments. This is especially true for extended practicals and project work.

KEY POINT An effective record of your practical work should:

- outline the purpose of your experiment or observation
- provide details of all materials and methods used
- record all relevant information about your results/observations
- provide an initial visual representation of the data
- note your immediate conclusions and any suggestions for further work.

Keeping a record – storage and retention of back-up copies of primary data from research projects and postgraduate work is often covered by a specific code of research conduct – check with your supervisor to see how this is handled at your institution.

Recording primary data – never be tempted to jot down data on scraps of paper: you are likely to lose them and you may forget what individual values mean.

Recording data in the laboratory

Although the traditional paper-based bound notebook (p. 215) is still favoured by some, laptop computers are now widely used for data recording. Computer-based recording offers several advantages, including ease of transfer of recorded data between applications for further analysis together with ease of back-up and storage.

While individual observations (for example, temperature) can be noted in text form, data collection tables are often the most appropriate way to record large amounts of information. It is often best to tackle this using a computer and spreadsheet; once prepared, you can either record the data directly into the cells of the spreadsheet or use a print-out for ‘hard copy’ recording. When preparing a table for data collection, you should:

1. **Use a concise title, or a numbered code for cross-referencing.** Including the date in your file name can be useful.
2. **Decide on the number of variables to be measured and their relationship with each other and lay out the table appropriately:**
 - (a) The first column of your table should show values of the independent (controlled) variable, with subsequent columns for the individual (measured) values for each replicate (‘rep’) or sample (Fig. 31.1(a)).

(a)

Level of controlled variable	Measured variable		
	Replicate 1	Replicate 2	Replicate 3
1			
2			
3			
4			

(b)

Treatment	Measured variable	
	Organism 1	Organism 2
A		
B		
C		
D		

(c)

Time	Measured variable	
	Control	Treated
1		
2		
3		
4		
5		
6		

Fig. 31.1 Example layouts of tables for recording data: (a) measurements on replicates at different levels of a controlled variable; (b) responses by two organisms to a range of treatments; (c) time course of responses in control and treated specimens. Note the grid-like nature of these tables used for ease of data entry compared with the 'horizontal lines only' style used for formal presentation of tables (Chapter 73).

Labelling your records – always ensure that your data table/notes include all potentially relevant details of the observation or experiment (including, where not recorded elsewhere, the date when the recordings were made).

Recording notes on paper – bear in mind the following advice:

- A bound notebook is your best option, as you are less likely to lose pages.
- If you need to use loose paper, make sure each sheet is dated and easily identifiable – either paste these into your notebook or file consecutively in a ring binder (the same applies to any traces, printouts and graphs produced by instruments).
- Write with a pencil, so that mistakes can be corrected easily.
- Write clearly, taking special care that individual numerals cannot be confused.
- Use a tally chart (p. 548) if recording events – this will also provide an 'instant' histogram of your results.

(b) If several variables are measured for the same organism or sample, each should be given a row (Fig. 31.1(b)).

(c) In time-course studies, each time point should be a separate row – put the replicates as columns grouped according to treatment, with the rows relating to different times (Fig. 31.1(c)).

- 3. Make sure the layout reflects the order in which the data values will be collected.** Your table should be designed to make the recording process as straightforward as possible, to minimise the possibility of mistakes. For final presentation, a different arrangement may be best, particularly where data values have been manipulated or transformed prior to presentation (Chapter 71).
- 4. Consider whether additional columns are required for subsequent calculations.** Create a separate column for each mathematical manipulation, so the step-by-step calculations are clearly visible. Spreadsheet software and formulae (Chapter 72) can help avoid mathematical errors for repetitive calculations.
- 5. Allow sufficient time within your experimental protocol to record quantitative data accurately.**
- 6. Record numerical data to an appropriate number of significant figures,** reflecting the accuracy and precision of your measurement (p. 187). Do not round off data values, as this might affect the subsequent analysis of your data.
- 7. Prepare blank copies of data recording tables if your experiments or observations will be repeated.**
- 8. Explain any unusual data values or observations in a footnote.** Do not rely on your memory.

Recording details of project work

The recommended system is one where you make a dual record.

Primary record

This is the initial record, made at the bench or in the field. In this, you must concentrate on the detail of materials, methods and results. Include information that might prove useful in error tracing: for example, if you note how a solution was made up (exact volumes and weights used rather than concentration alone), this could reveal whether a miscalculation had been the cause of a rogue result. Note the origin, type and supplier of

Using data loggers/recorders – these record data from an instrument at defined intervals and store the information temporarily in an electronic memory ('log'). These primary data can be downloaded to a computer either directly or at a later point. Advantages include the benefit of automatically collecting measurements at a speed not feasible for individual lab workers, enabling a comprehensive data set to be accumulated. When using a data logger, you should make back-up copies of each period's data as soon as possible, otherwise information may be lost if the recording instrument's memory is cleared or overwritten, or if the machine fails.

Using electronic laboratory notebooks – a number of companies now offer software products for data recording in labs (e.g. LABtrak). However, these are mainly used by industrial companies to assist them to comply with regulations assuring quality and data security. For student purposes, standard software such as a word processor, spreadsheet and possibly a database should be satisfactory.

Considering formal aspects of keeping a record – the diary aspect of a record can be used to establish precedence (e.g. for patentable research where it can be important to 'minute' where and when an idea arose and whose it was); for error tracing (e.g. you might be able to find patterns in the work affecting the results); or for explaining your activities to a supervisor.

any chemical(s) and organism(s) used. Make rough diagrams to show the arrangement of replicates, equipment, etc.

The basic order of the primary record should mirror that of a research report (see p. 80), including: the title and date; brief introduction; comprehensive materials and methods; the data plus any initial analysis; and short conclusions.

When creating your primary record, take care not to lose any of the information content of the data: for instance, you should record every individual data measurement, rather than just a mean value, as this will enable you to use statistics to describe the variability within the data set (Chapters 76 and 77).

Secondary record

Use your primary record to produce a secondary record that is better organised and presented. This should be in electronic format (for example, a file produced using a word processor). Your secondary record will be used when discussing results with your supervisor, and when writing up your report or thesis – in some instances, this record may be part of your course assessment. Although the text should retain the essential features of the primary record, it should be more concise and the emphasis should move towards *analysis* of the experiment or observation. Outline the aims and objectives at the start and link the data set to others in a series (for example, 'Following the results of Expt D24, I decided to test whether . . .'). You should present data in an easily understood format, for example, as tables of means or as summary graphs. Use appropriate statistical tests (Chapter 77) to support your analysis.

The dual method of recording outlined above deals with any limitations of hard-written notes taken at the bench or in the field. Producing a second, neater version forces you to consider again details that might have been overlooked in your primary record and provides a duplicate in case of loss or damage.

If you find it difficult to decide on the amount of detail required in Materials and Methods, the basic ground rule is to record enough information to allow a competent scientist to repeat your work exactly. You must tread a line between the extremes of including pedantic fine detail and the omission of information essential for a proper interpretation of the data – better perhaps to err on the side of extra detail to begin with. An experienced lab worker can help you decide which subtle shifts in technique are important (for example, batch numbers for an important chemical, or when a new stock solution is made up and used). Many scientific advances have been made because of careful observation and record taking or because coincident data were recorded that did not seem of immediate value.

There are many ways to reduce the labour involved in keeping a record. Do not repeat Materials and Methods for a series of similar experiments; use devices such as 'method as for Expt B4', or give each method a unique name/code. A photocopy might be sufficient if the method is derived from a published source (check with your supervisor). To save time, prepare blank checklists, worksheets and tables for future use.

Using communal databases to record research information

When carrying out project research as part of a research team, you may need to use their communal databases for recording purposes. These avoid

Analysing your data as soon as possible – always consider your data immediately after collection as this may influence your subsequent activities.

- Generate graphs of your data, since these can be particularly valuable for indicating trends, highlighting differences, etc.
- Carry out statistical analyses (Chapter 77) before moving on to the next experiment because apparent differences among treatments may not turn out to be statistically significant when tested.
- Write down any conclusions you make while analysing your data: sometimes those that come to mind at the time of doing the work are forgotten when the time comes to write up a report or thesis.
- Note ideas for further studies as they occur to you – these may prove valuable later. Even if your experiment appears to be a failure, suggestions as to the likely causes might prove useful.

SAFETY NOTE Maintaining and consulting communal lab records – these activities may form a part of the safety requirements and quality standards for working in a laboratory.

duplication of effort and ensure uniformity in techniques. You will be expected to use the databases carefully and to contribute to them properly. They might include:

- **a shared file of common techniques** (for example, how to prepare growth media or solutions) – you may be asked to add your own methods to this listing
- **a set of simplified step-by-step instructions for use of equipment** – manuals are often complex and poorly written and it may help to redraft them, incorporating any differences in procedure that have been adopted by the group
- **an alphabetical list of suppliers of equipment and consumables**, to which you could add
- **a list of chemicals required by the group** and where they are stored – here, you might be expected to record what you use, to help with reordering
- **the risk-assessment sheets for dangerous procedures** (pp. 127–8) – you will need to consult these, and add to them for new techniques
- **the record book detailing the stock of radioisotopes and their disposal** – here, you must record your usage.

Using photography and imaging to record information

These techniques may be required within your degree program for the following:

- **documentation and validation of observations** for coursework or projects (Chapter 32)
- **recording visual information** for later measurement, counting and/or analysis (Chapter 71)
- **creation of material to illustrate talks** (Chapter 16)
- **preparation of content for poster displays** (Chapter 15)
- **producing ‘hard copy’ of microscope images** for project work (Chapters 25 and 26)

For biomolecular sciences research, photography is usually carried out in two main situations:

1. **Laboratory or studio environments** – where lighting conditions are under your control and problems are relatively easy to overcome.
2. **Work with specialised equipment** – such as photomicroscopes or electron microscopes, where control of lighting and the photographic process is largely managed by the instrument’s operating system (p. 175).

Images can be acquired using different forms of camera or by scanning. Most cameras and scanners use digital technology. However, for some specialised purposes, film cameras may be preferred. Cameras operate by capturing the ambient light reflected by objects, while scanners generally sense the reflected light from an integrated lamp as it moves in front of the object. Some methods such as thermal imaging capture the radiation emitted directly by an object. For certain studies, videography will be appropriate: this involves taking a series of images which can then be viewed in sequence (see Jones *et al.*, 2021).

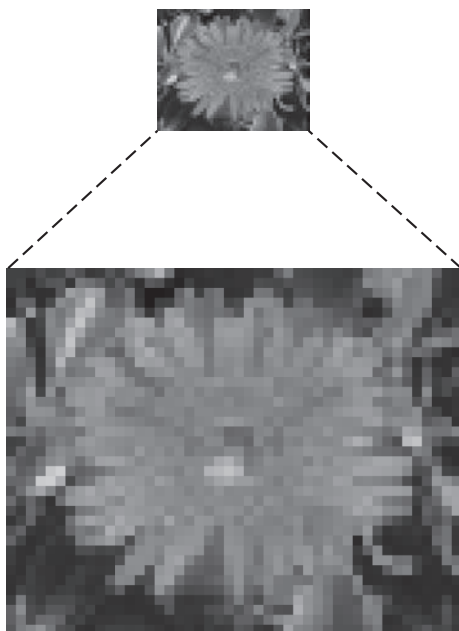


Fig. 31.2 Pixel structure in a digital image. When close detail of a digital image is examined, the pixellated nature of the image is often revealed.

Understanding digital camera sensors –

these are complex solid-state devices built of microcircuits on a silicon wafer. Small wells (pixels) contain light-sensitive elements that produce an electrical response when hit by light. The signals are then amplified and converted into digital form which can be processed and stored by a computer chip. Filters are used to provide colour information.

Cleaning camera lenses –

dust particles and grease can adhere to the outer glass of the image sensor or camera lens and reduce the quality of images by causing spots or blurred regions on images. Great care should be taken when cleaning lenses, using specialised wipes or cloths, otherwise permanent damage can occur from scratching or removal of the delicate lens coating.

KEY POINT Although you may be familiar with photographing digital images in social situations, e.g. with your smartphone, understanding some of the technical aspects of photography will allow you understand relevant terminology and take better pictures that are suitable for scientific purposes.

An electronically stored image, produced using either a digital camera (still or video) or a scanning device from a pre-existing image, is recorded as a series of discrete picture elements called pixels (Fig. 31.2). The greater the number of pixels per unit surface area, the better the potential resolution of the image and the greater the amount of detail captured. You can record the image in black/white, greyscale or colour. Image resolution is constrained by the nature of the sensor array and the amount of memory available for recording, while size and quality will also depend on the display or printer used.

Understanding camera lens systems

While smartphones and compact digital cameras have their uses, higher-quality images can be taken with a single lens reflex (SLR) camera (Fig. 31.3). SLR refers to the retractable mirror system that allows the user to preview what is recorded using either a screen or directly through the lens (TTL). SLR cameras usually have better-quality and interchangeable lenses that allow greater control over the image recorded. Lenses are either fixed, with a set focal length, or are able to zoom, allowing a range of focal lengths, and hence magnification and angle of view (Fig. 31.4). Focal length is important to consider because it defines the angle of view and magnification of the image. Thus, a wide-angle lens will have a low focal length (for example 28 mm) while a telephoto lens will have a high value (for example, 200 mm). The optics and mechanical systems of lenses can be extremely complex, especially for zoom lenses, so the best quality types are usually expensive.

To use an SLR camera properly, you need to understand the relationship between aperture (f-number), shutter speed and depth of field. The f-number refers to the lens aperture (opening), which defines the amount of the image that appears in focus – the higher the f-number, the smaller the aperture and the greater the range of focus. For convenience, this range is



Fig. 31.3 A typical SLR digital camera.

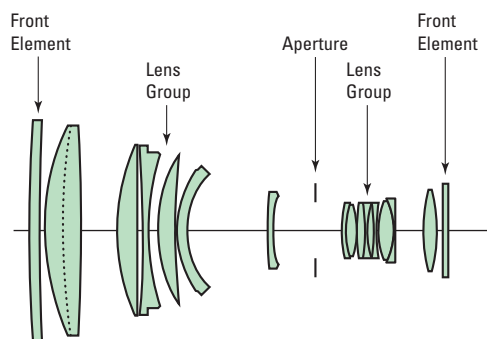


Fig. 31.4 Cross-sectional diagram of optics within a zoom lens. The zoom effect is achieved by movement of the lens groups.

Focusing images correctly – the autofocus feature on cameras may result in lack of focus on the parts of the image you are interested in because the algorithm used (generally spot or area) may not be suitable for your purpose. Better results may be obtained using manual focusing.

Understanding the light sensitivity of photographic systems – both digital and film media are rated in terms of their ‘speed’, measured in either ISO (= ASA) or DIN units. A good range for general purposes is 100–160 ISO (21–23 DIN). Film of 1600 ISO is available, as are digital cameras with sensor settings as high as 3200 ISO. These are useful for low light situations, but with some loss of image quality.

usually indicated on lens sleeves. Fast shutter speeds allow the recording of moving objects with less blur. F-numbers and shutter speeds are inversely related, but in SLR cameras, this relationship can usually be controlled by the photographer. Thus, for moving objects, you should give priority to fast shutter speed by opening the lens aperture (small f-number). Where depth of field is required, you should close the lens aperture (high f-number); this will result in slower shutter speeds, so take care to avoid camera shake. It is good practice to ‘bracket’ your exposures (make extra exposures at plus and minus 1 f-number at the same shutter speed) to ensure good results.

Taking account of lighting conditions

The amount and type of light available to create an image are very important, especially for colour photography. The ‘colour temperature’ of a light source is measured in Kelvin. You may therefore need to adjust your camera settings for the appropriate light temperature: the main options are for ‘tungsten’ (indoor) lighting, fluorescent lighting or outdoor (sun) light. In most digital cameras light quality is automatically sensed, but can be overridden where necessary using ‘white balance’ settings.

The quantity of light can be measured by a photographic light meter, which may be external or built-in. The more light there is, the smaller the lens aperture you can select (larger f-number), and the greater will be the depth of focus. With the camera on a tripod, and for a given sensor sensitivity, you can select slow shutter speeds and larger aperture settings (f-numbers), to maximise depth of focus. Where an electronic flash system is employed to provide some or all of the lighting this is even easier, since the effective shutter speed with electronic flash is extremely short (1 ms). Alternatively, you may wish to select a faster shutter speed to obtain a narrower depth of focus to highlight a specific item on the image.

KEY POINT Your choice of lighting arrangement will affect the quality of the picture. Shadowless lighting is appropriate in some situations, but often shadows help to give three-dimensional form to subjects. In general, the use of a diffuse light source, or more than one source, is advisable to prevent hard shadows.

Photomicrography

This involves the use of a camera system mounted on a microscope (Chapter 26). The light source is built into the microscope, and the image captured is one of transmitted light, as affected by the specimen’s preparation, particularly its staining.

To get the most out of photomicrography, you should:

- 1. Carefully prepare your specimen.** Ensure thorough cleanliness of any slides or coverslips used.
- 2. Choose the correct camera settings** and any filters required for alteration of colour balance, depending upon the type of light source available.
- 3. Decide on the magnification to be used:** make sure that you know how this relates to the magnification of the image. Always include a photograph of a stage micrometer (p. 181) so the final magnification

Examples Lighting systems for photography include:

Natural daylight – variable in nature and can involve shadowing.

Indoor (artificial) lighting – may not be bright and can cause difficulties due to differences in colour temperature compared with natural daylight.

Camera flash – inbuilt flash systems may lack the power to effectively light the subject and their unidirectional nature may result in shadowing or otherwise 'harsh' images.

Independent flash – these are linked to the camera but may be more powerful and ring or panel systems can provide a diffuse light to avoid shadowing.

Backlighting – semi-transparent subjects like electrophoresis gels may be placed on a 'lightbox' so the image highlights lighter and darker bands (Chapter 50).

Improving your photography – the following tips may be useful:

- To minimise camera shake, use your camera on a tripod whenever possible, and/or use a faster shutter speed. Otherwise, adopt a posture with your elbows firmly locked against your body.
- As far as possible, consider composition so that you include all relevant features of the item of interest.
- For uniformity of colour balance, use an electronic flash wherever possible.
- To avoid shadowing, reflections and overexposure, use a ring-flash or panel lighting system, or use a tripod with slower exposure speed under natural light.

Cropping images – take care to 'lock' the aspect ratio (the ratio of height to width), to avoid distortion of the image when you change its framing.

can be calculated and given in the legend to any photographs used in your report.

4. Carefully focus the specimen onto the image plane.

5. Bracket extra exposures above and below the automatic settings.

For more detailed guidance, consult the instruction manual for your photomicroscope.

Scanning

Scanners can be useful to digitise a printed image, or to create an image of a flat object, such as an electrophoresis gel (if placed in a plastic bag beforehand). In flatbed scanners, you place the source image or item on a glass plate and a mobile lamp traverses the item, first to allow the system to analyse of its size and characteristics, then to allow a charge-coupled device (CCD) array to capture the image using mirrors and lenses. Filters are used to create colour images. The quality of the scanned image is defined in terms of the number of dots per inch (dpi). Most printers incorporate a scan feature, and a scanner with a value of 600 dpi is adequate for most purposes. For scanned images, resolution is usually greater than 6 megapixels (up to 25 megapixels, depending on scanner).

KEY POINT Take care when copying images from other sources – you may be at risk of committing plagiarism and copyright infringement (Chapter 5).

Image processing

Digital cameras record images as files on various forms of memory cards; the data can later be transferred either to a computer for manipulation or directly to a printer. An appropriate card reader or cable may be required. For critical work, you should try to capture images using the largest possible file size, as you can reduce digital information later, if necessary (that is, compress file size), but you cannot add back information that is not captured at the outset.

Digital image files can be stored in various formats (for example, .bmp, .jpg, .tiff) and can be manipulated using programs such as Picture Manager (Microsoft Office), Adobe Photoshop or PaintShop Pro. Aspects of image quality can be adjusted, including:

- **image framing and format (cropping)**
- **brightness and contrast**
- **colour balance**
- **aspect (image rotation)**
- **file size**

KEY POINT The ease with which digital images can be manipulated can lead to problems because an adjusted image may not represent the true and original situation. For scientific work, you should employ the original, unchanged image whenever possible – although some adjustments to image quality such as contrast may be valuable and acceptable.

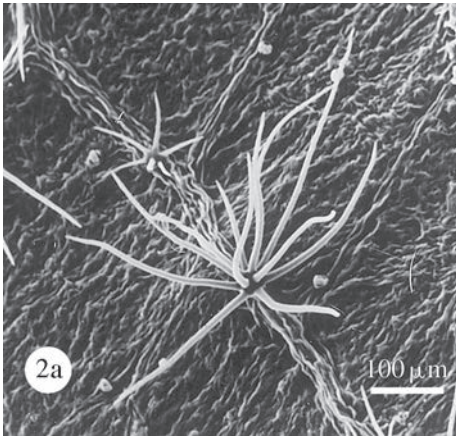


Fig. 31.5 Use of white areas for labelling dark prints, illustrated by an SEM micrograph of a stellate hair on a leaf surface.

Using scales with images – these are vital to the interpretation of any biological image (see Chapter 25 for information on different types of scale and how to calculate them).

Recording information about images – this should include subject, location, image-capture details and processing procedures. Bear in mind that these data may be of use when the image is used in a different context from the one you initially planned.

Backing up digital image files – since no physical copy exists, you must remember to download and back up digital image files to appropriate media (cloud storage, hard drive, network drive, DVD or USB memory stick) as soon as possible.

Printing

While you can easily incorporate digital images into word-processor or presentation files, you may wish to produce prints to ensure the highest level of image quality. Choose your print type depending on your purpose. Gloss-finish prints generally appear sharper than matt or other finishes, and are probably best for project reports (Chapters 12 and 33). Moreover, the addition of lettering and scales is often only possible on the smooth surface of a glossy print. However, when preparing prints for display on a poster (Chapter 15), matt/velvet finishes are often preferable, as there is less glare for the viewer. When used for display, mount prints on stiff board either with modern photographic adhesives or dry-mounting tissue.

Printing from computers using ink-jet and similar printers requires careful checking of colour balance, since colours viewed on monitors do not always reproduce on paper as expected. You may need to experiment with settings and paper quality to obtain the appropriate results.

Adding scales and labels

Having created a suitable image, it is often necessary to add information to it. When digital images are copied into word-processor or presentation software, additional information such as lettering, scales and labels can be added by overlaying a ‘text box’, or equivalent. You may need to make the background transparent, for example, using the ‘no fill’ option in *PowerPoint*. Lettering can be added to printed media using transfer letters (for example, Letraset). Choose a simple font type and a size that is legible but not too dominant. Always use the same font and type size for a related set of images. Select an appropriate part of the image for the lettering, for example, a dark area for a white letter. If you cannot find an area of the photograph with a suitable, even tone, create a coloured or white area and place the letters on this (see Fig. 31.5).

Image storage

Cloud computing (for example, *Apple iCloud*, *Microsoft OneDrive* and *Google Drive*) allows image catalogues to be stored remotely (and securely), allowing access from multiple platforms that are independent of your location. Otherwise, ensure that any files of images are backed-up frequently, with storage media held in multiple locations. Indexing software can be used to organise and catalogue your images (for example, *Picasa*, available for free download at: <http://picasa.google.com/>). These programs allow you to add appropriate information and store this electronically along with the image, allowing viewing and selection when your collection of images is large. Prints should be stored flat in boxes or in albums.

Text reference

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Sources for further study

Anon. *Suggestions for Keeping Laboratory Notebooks*. Available: <https://otl.stanford.edu/suggestions-keeping-laboratory-notebooks> Last accessed 11/03/21. [A Stanford University website that looks at the laboratory notebook from the patenting perspective.]

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STUDY EXERCISES

31.1 Design a primary data collection sheet for a lab experiment. Imagine you wish to count and identify the different types of microbes present in water and able to grow on nutrient agar at 25 °C. What might you need to record on your data sheet?

31.2 Outline the advantages and problems that would be associated with using a digital audio recorder to record data.

31.3 Design a secondary record table for the collection and analysis of a set of count data for the number of bacterial colonies developed in each of ten replicates for each of five different treatments. Assume that you need to calculate means, variances, etc., and then to compare the results for each treatment.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

32 Tackling project work and placements

Obtaining ethical approval – if any aspect of your project involves work with human or animal subjects, then you *must* obtain the necessary ethical clearance before you begin; consult your department's ethical committee for details (see Chapter 30).

Using the Internet as an information source – searches using relevant key words may indicate institutions where research in your area is currently being carried out, and the names of key researchers. Academic staff usually respond positively to emailed questions about their area of expertise.

Asking around – one of the best sources of information about supervisors, laboratories and projects is past students. Some of the postgraduates in your department may be graduates from your institution and they could provide an alternative source of advice.

Research projects are an important component of the final-year syllabus for most degree programmes in the biomolecular sciences, while shorter projects may also be carried out during courses in earlier years. Placements involve periods working in organisations whose activities are relevant to your degree. Both project work and placements can be extremely rewarding, although they do present a number of challenges in terms of acquiring specialised knowledge and skills, working in a research/industrial environment and meeting the expectations of researchers or work supervisors. The assessment of your project or placement is likely to contribute significantly to your degree grade, so all aspects of this work should be approached in a thorough manner.

Deciding on a project topic

Assuming you have a choice, this important decision should be researched carefully. Visit possible supervisors and ask them for advice on any offered topics that you find interesting. Use library texts and research papers to obtain further background information. Perhaps the most important criterion is whether the topic will sustain your interest over the whole period of the project. Other things to look for include:

- **Opportunities to learn new skills.** Ideally, you should attempt to gain experience and skills that you might be able to 'sell' to a potential employer.
- **Likelihood of obtaining interesting/valid results.** An ideal project provides a means to obtain useful data to help you construct a report, but also the chance to extend knowledge by doing genuinely novel research.
- **Assistance.** What help will be available to you during the project? An active lab with many research students might provide a supportive environment should your potential supervisor be too busy to meet you often; on the other hand, a smaller lab may provide the opportunity for more personal interaction with your supervisor.
- **Impact.** Your project may result in publishable data: discuss this with your prospective supervisor.

Writing a project proposal

You may be required to submit a proposal for your project, especially where it is expected that you should define the precise area of study yourself. The structure for the proposal will probably be provided in the course handbook, or as a form you must complete. Box 32.1 outlines some common features of project proposals and what you should do to complete each part.

The aim of formulating a proposal is to ensure that:

- **the project has an appropriate theoretical background**
- **the objectives you have set are achievable**
- **the methods chosen are appropriate**
- **safety and ethical issues have been considered**
- **sufficient resources are available to complete the work**, such as matching students to available labs and supervisors
- **you have set yourself a timetable with milestones** on which your progress can be judged
- **you obtain feedback and suggestions about your plans.**

Box 32.1 How to write a project proposal

Below are listed some common elements of project proposals, with guidance regarding approach and content. Not every part may be required, and the titles for sections may differ – always follow the exact format specified for your course.

- **Contact details and suggested supervisor(s)** – you may be asked whether you have discussed the project with the named supervisor(s).
- **Proposed title** – this should be relatively short, following the style used in research papers. It may change for the final report.
- **Aims** – a general statement of what you plan to achieve.
- **Objectives** – a listing of specific outcomes you expect to fulfil. Typically you will have several specific objectives that all fit within the overall aim.
- **Brief description of the subject** – this section might have an alternative title such as Summary, Background, Review of Subject Area or Statement of the Problem to be Addressed. It should contain a brief synopsis of past work, a summary of current ideas and, if relevant, the hypothesis to be tested. In some cases it will consist of a mini-review of the subject area and act as a template for the introduction to your report.
- **Preliminary bibliography** – this will ensure (a) that you have read and understood relevant papers and (b) that you understand how to cite them properly (Chapter 5).
- **Research methods** – describes how you plan to carry out the investigation. Be quite specific, so the committee can arrive at a valid judgement. Quote references for methods and techniques, where available. Explain your experimental design, including controls (Chapter 29). Ensure this does not result in a page over flow. Check that the results obtained will tell you what you need to know to achieve your objectives and to test your hypothesis.
- **Resources required** – the samples, chemicals, instruments, etc. required to carry out your investigation. Listing these will require quite detailed consideration of the experimental design or field area, as relevant, and a thorough knowledge of methods, such as amounts or volumes of reagents used (Chapters 22 and 23).
- **Timetable/plan** – a realistic breakdown of the work required, with milestones leading to completion of the project (see Chapter 2 for advice on time management). Always allow time for the unexpected.
- **Statement or declaration in relation to safety and ethical rules** – this is to confirm that you have read and understood relevant issues, have completed relevant forms and processes and are in a position to proceed with the research. You may be required to attach copies of COSHH forms (Chapter 20) or ethics committee permission (Chapter 30).

Your proposal outline might be considered by your supervisor or by a formal committee before approval.

Planning project work

As with any lengthy exercise, planning is required to make the best use of the time allocated (Chapter 2). Figure 32.1 illustrates a potential division of

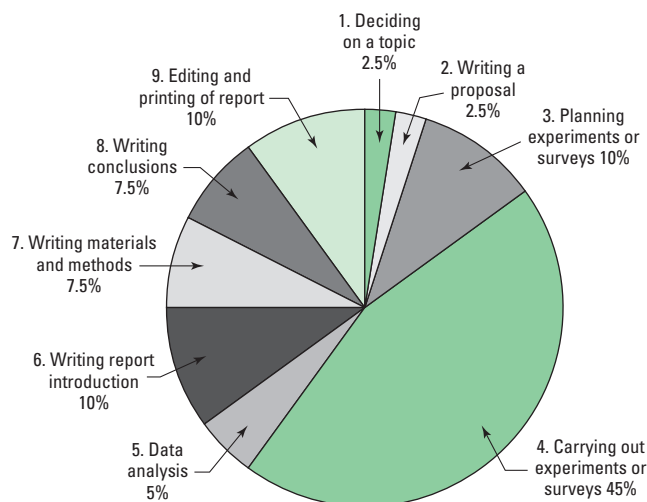


Fig. 32.1 Pie chart showing representative time allocations for different phases of a final-year project.

Writing an interim report – if you are required to do this, look on the task positively as an opportunity to clarify your thoughts and get some of the time-consuming preparative work out of the way. If not, you should set your own deadlines for producing drafts of the introduction, materials and methods section, etc.

time for a typical project. For convenience, the different phases are expressed as a proportion of the total time available, but in practice they should overlap to make the best use of time. This can be illustrated in a Gantt chart (Fig. 32.2), which may also be used for tracking progress. Even this format does not tell the whole story though, as science is an iterative process with unpredictable outcomes, and hence your plan should be flexible to allow you to respond to such outcomes. However, where projects are concerned, the available time is always limited and you will need to use this wisely and plan when to stop researching and complete your dissertation or thesis.

While effective and focused planning like this is valuable, it is important not to devote too much time to planning itself, rather than carrying out the work required to achieve results and to write them up – you need to strike a balance. It is especially important not to underestimate the time it will take to write and produce your thesis (see below). Since a large proportion of marks will be allocated to the report, you should not rush its production. If you wish to benefit from feedback given by your supervisor, you should aim to provide drafts to them in good time.

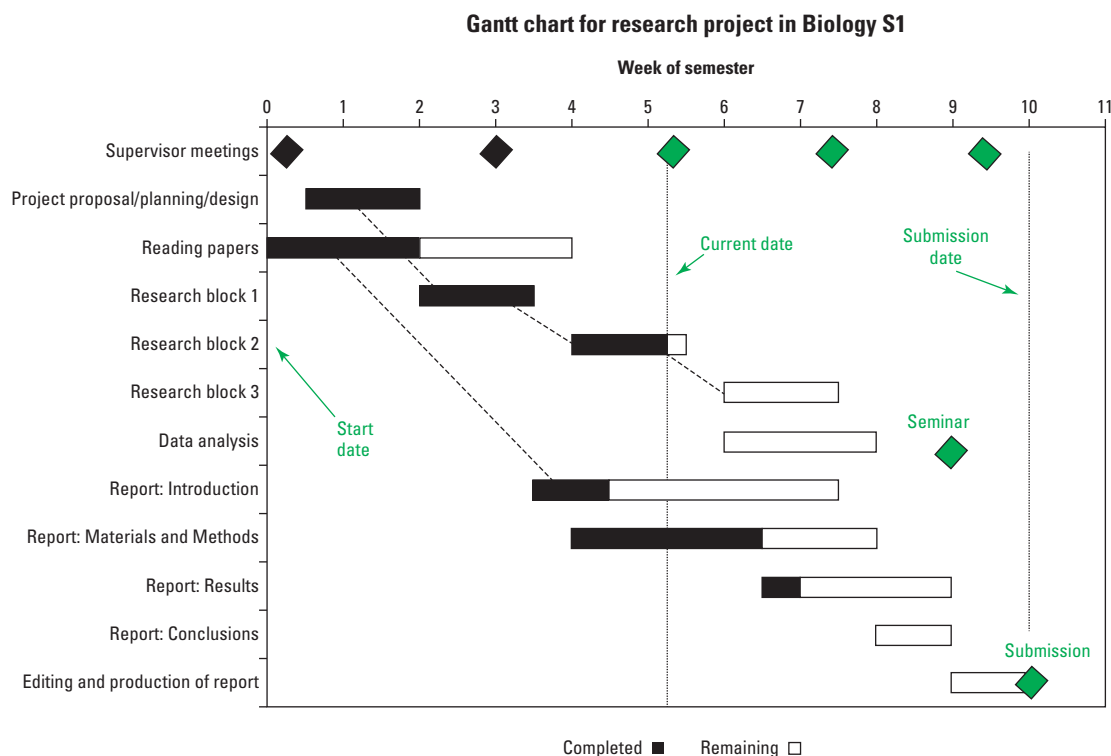


Fig. 32.2 Gantt chart for project work. This chart was created using the 'stacked bar' type of chart in Excel and illustrates progress at the start of the 5th week of a representative 10-week research project. Note how phases of work are shown as overlapping, indicating, for example, that work can proceed on writing a report introduction or materials and methods section while still carrying out experimental work. The latter is shown in 'blocks' reflecting the student's access to relevant lab facilities. Progress in each phase is shown by shading in the horizontal bar for each element: this student is shown as 'behind schedule' with the report's Introduction, but they have started to write parts of the Materials and Methods section, so are 'ahead of schedule' on this aspect. Connections between elements are shown with a dotted line, such as the obvious need to complete the design of experiments before conducting them, or read relevant papers before citing them in the Introduction. Key milestones in the plan (shown as lozenges) include meetings with the student's supervisor, a seminar to be presented at the end of week 8 and the final report to be submitted in week 10.

Liaising with your supervisor(s) – this is essential if your work is to proceed efficiently. Specific meetings may be timetabled, e.g. to discuss a term's progress, review your work plan or consider a draft introduction. Most supervisors also have an 'open-door' policy, allowing you to air current problems or discuss your results. Prepare well for all meetings: have a list of questions ready before the meeting; provide results in an easily digestible form (but take your lab notebook along); be clear about your future plans for work.

KEY POINT Project work can be very time-consuming. Try not to neglect other aspects of your course – make sure your lecture notes and necessary reading/note-making are up to date as you go along.

Getting started

At the start of your project, do not spend too long reading the literature and working out a lengthy programme of research. Get stuck in: make some preliminary observations, do a simple experiment or conduct an initial survey. There is no substitute for 'getting your hands dirty' for stimulating new ideas:

- **even a 'failed' experiment or survey will provide some useful information** that may allow you to create a new or modified hypothesis
- **pilot observations and experiments may point out deficiencies in experimental technique** that will need to be rectified
- **preliminary questionnaires may allow you to design better ones** or refine the precise area you wish to survey
- **the experience will help you create a realistic plan of work.**

Planning experiments and developing sampling procedures

Design of experiments is covered in Chapter 29. Avoid being too ambitious at the start of your work. It is generally best to work with a simple hypothesis and design your experiments or sampling around this. A small pilot experiment or test sample will highlight potential stumbling blocks, including resource limitations, whether in materials, or time, or both.

Designing and conducting surveys

Some projects may involve surveys of the public or a sub-set of it. For example, it may be of interest to gauge attitudes to a conservation project, to an area of scientific research such as molecular genetics or to a medical intervention like vaccination. Surveys can be of two main types:

Taking account of the ethical and data protection aspects of surveys – you must follow your university's guidelines carefully (see Chapter 30). You will need to:

- inform participants about the purpose of the study
- obtain signed consent for recording their input and for the use of their responses in your research
- store confidential data and recordings of participants appropriately and for a limited period (p. 212).

- **Questionnaire-based surveys and interviews.** These are generally based on 'closed' styles of questions where the participant must select from pre-defined options (for example, 'yes/no' or multiple-choice designs, pp. 112–14). The responses are relatively easy to quantify. Face-to-face survey interviews often result in more considered responses than paper-based or computer-delivered questionnaires.
- **Focus groups.** Here, organised by a moderator, the participants are asked to discuss a topic, often in response to an 'open' question or statement, and the outcome of their dialogue is recorded. This reduces the possibility of opinions being 'led' by a question-setter, but results tend to be qualitative in nature and difficult to summarise.

Box 32.2 provides some general guidance for conducting surveys.

Box 32.2 How to design a questionnaire and conduct a survey

A. Designing the questionnaire. Observe the following principles:

- **Keep the survey as short as possible** – do not waste your own and participants' time by asking irrelevant questions.
- **Obtain appropriate demographic information.** Collect data that might be useful to correlate with the answers obtained (see C. below).
- **Only ask a question if you have clear idea of how you will use the information obtained.**
- **Try to ask 'neutral' questions.** Beware slanting the wording to encourage the type of response you would like (e.g. that might support a favoured hypothesis).
- **Choose question styles that will yield useful information** – for example, these may be yes/no; multiple choice (pp. 112–14) or 'Lickert-scale' (Fig. 32.3) in response to a statement. Open question styles may be valuable and you might wish to include '*none of the above (please specify)*' as an option. While answers to questions that require quantitative or ranked answers may be easier to summarise or report, they will lack the detail and undirected nature of responses to questions requiring a qualitative answer. The latter are more difficult to condense and describe, but depending on the purpose of the survey, may provide more revealing answers.
- **Try to vary the types of question you ask** – to provide results appropriate to your needs (and to discourage participants from providing 'rote' answers).
- **Make sure your question wording is unambiguous** – you may have a clear idea of your topic, its terminology and the type of response you are seeking, but your respondents may not.
- **Move from general questions to those that are more specific** – this will reduce the possibility of leading the respondent by putting ideas into their mind.
- **Always pilot your survey before using it for research purposes.** This may help you to iron out problems and help estimate the time participants need to allocate for responding.

B. Selecting participants. This is less easy than it might seem and is often cited as a reason why some survey results do not represent the whole population. Consider

potential biases in your selection criteria or methods and how these might impact on the reliability or relevance of the conclusions you can draw. Ensure you ask enough people to be able to draw reliable conclusions; consult with your supervisor to decide how many this should be.

C. Using demographic information. This will allow you to partition results among different groups and may assist in accounting for bias in participant selection. Categories that might be of interest are gender, age, ethnic origin, educational attainment and employment. It is difficult to predict what information might be useful at the outset, but try not to waste your own and respondents' time by collecting obviously irrelevant data.

D. Analysing and presenting results. Remember that you will need to specify all of the above procedures in your methods section:

- **state your objectives and sampling method clearly**
- **provide a summary of demographic information of respondents**
- **explain the design of the questionnaire and include a copy**
- **outline how the survey was conducted**
- **summarise quantitative responses using appropriate descriptive statistics (Chapter 76)**
- **make sure any qualitative responses quoted are representative** (it is acceptable to correct respondents' grammar and spelling).

'Plastic bags should be replaced with paper bags in all supermarkets, to reduce waste and pollution'

Which of the following best describes your feelings about the above statement? (Circle the appropriate number.)

1. agree strongly
2. agree
3. neither agree nor disagree
4. disagree
5. disagree strongly

Fig. 32.3 Example of a Lickert scale question. These formats are designed to give answers that can be analysed quantitatively, e.g. '76% of respondents ($n = 35$) strongly agreed or agreed with. . . .'

Working in a laboratory

During your time as a project student, you are effectively a guest in your supervisor's laboratory.

- **Be considerate** – keep your 'area' tidy and offer to do your share of lab duties such as calibrating the pH meter, replenishing stock solutions, distilled water, etc., maintaining cultures, tending plants or animals.
- **Use instruments carefully** – they could be worth more than you would think. Careless use may invalidate calibration settings and ruin other people's work as well as your own.
- **Do your homework on techniques you intend to use** – there is less chance of making costly mistakes if you have a good background understanding of the methods you will be using.
- **Always seek advice if you are unsure of what you are doing.**

SAFETY NOTE It is essential that you follow all the safety rules applying to the laboratory or field site (Chapter 20). Make sure you know all relevant procedures – normally there will be prominent warnings about any hazards. If in doubt, ask.

Analysing and interpreting your results

Tidy record keeping is often associated with good research, and you should follow the advice and hints given in Chapter 31. As you obtain results, you should always calculate, analyse and graph data as soon as you can (see Fig. 32.1). This can reveal aspects that may not be obvious in numerical or readout form. Do not be worried by negative results – these can sometimes be as useful as positive results if they allow you to eliminate hypotheses – and do not be too dispirited if things do not work first time. Thomas Edison's maxim 'Genius is one per cent inspiration and ninety-nine per cent perspiration' certainly applies to research work.

Writing your project report

The structure of scientific reports is dealt with in Chapter 12. The following advice explains how you can collect relevant information for each section.

Introduction

This is a big piece of writing that can be very time-consuming. Therefore, the more work you can do on it early on, the better. You should allocate some time at the start for library work (without neglecting your field or bench work), so that you can build up a database of references (Chapter 5). You will find it valuable to have downloads or copies of key reviews and references handy when writing away from the library. Discuss proposals for content and structure with your supervisor to make sure your effort is relevant. Leave space at the end for a section on aims and objectives. This is important to orientate readers (including assessors), but you may prefer to finalise the content after the results have been analysed.

Materials and methods

You should note as many details as possible *when doing the experiment or making observations*. Do not rely on your memory or hope that the information will still be available when you come to write up. Even if it is, chasing these details can waste valuable time.

Results

Show your supervisor graphed and tabulated versions of your data promptly. These can easily be produced using a spreadsheet (Chapter 72),

Brushing up on your IT skills – while Chapters 14 and 72 detail key features of word processors and spreadsheets, you may feel you could benefit from attending relevant IT courses or studying guidebooks so that you can use them more efficiently.

Using drawings and photographs – these can provide valuable records of sampling sites or experimental set-ups and could be useful in your report. Plan ahead and do the relevant work at the time of carrying out your research rather than afterwards. Refer to Chapter 31 for tips on technique.

but you should seek your supervisor's advice on whether the design and print quality is appropriate to be included in your report. You may wish to access a specialist graphics program to produce high-quality graphs and charts: allow some time for learning its idiosyncrasies. If you are producing a project poster for assessment (Chapter 15), be sure to mock up the design well in advance. Similarly, think ahead about your needs for any seminar or poster you will present.

Discussion

Because this comes at the end of your report, and some parts can be written only after you have all of your results in place, the temptation is to leave the discussion to last. This means that it might be rushed – not a good idea because of the weight attached by assessors to your analysis of data and thoughts about future experiments. It will help greatly if you keep notes of aims, conclusions and ideas for future work as you go along (Fig. 32.1). Another useful tip is to make notes of comparable data and conclusions from the literature as you read papers and reviews.

Acknowledgements

Make a special place in your notebook for noting all those who have helped you carry out the work, for use when writing this section of the report.

References

Because of the complex formats involved (Chapter 5), these can be tricky to type. To save time, process them in batches as you go along, or use a database such as EndNote (p. 37).

KEY POINT Make sure you are absolutely certain about the deadline for submitting your report and try to submit a day or so before it. If you leave things until the last moment, you may find access to printers, photocopiers and binding machines is difficult.

Working on a placement

Many universities operate student placement schemes where you may be given the opportunity to carry out work or research in a subject-related industry or public body. Academic credit will normally be given for work in placement, according to the specific schemes operated by your university. Engage with the allocation process at the earliest opportunity, and make sure you fully understand the aims and objectives of the placement together with any project activities that might be included, and how you will be assessed; study the intended learning outcomes and bear these in mind while at work. You should also have a clear idea of how your placement will be assessed, and make relevant notes as you go through the experience. In most cases you will be expected to write a report reflecting on your experiences – this is likely to form part of your assessment.

Dealing with placement interviews –

allocation to some organisations may be competitive, and these may involve interviews at potential workplaces.

Prepare well for these. For example:

- find out about the company and the job beforehand
- have a clear and forward-looking view of what you want to achieve
- be ready for interview challenges, such as 'What if?' questions
- think carefully before answering questions
- dress suitably and have a positive attitude.

Creating personal objectives for your placement –

think through what you hope to gain from the opportunity. It may be useful to think about both short- and long-term aspects. For example, in the first few weeks or months you will probably hope to learn relevant skills, while by the end of the work period, you will possibly want to focus on your ambitions for future employment. Be prepared to communicate your objectives to supervisors.

Saying 'thank you' – at the end of a placement, do not forget to write or email to thank those who have helped you along the way.

Approach your placement with the following in mind:

- **Carry out some research on the company or organisation beforehand.** This background information will help you understand its origins, scope of work and, importantly, its objectives, vision or mission.
- **Remember that first impressions count.** At initial meetings or interviews, show you are up-beat about the opportunity the organisation is giving you. Take notes of things that could be important later, for example, people's names and roles and aspects of the organisational structure.
- **Follow all workplace safety rules during your placement.** These are there for a reason and failure to observe them may be a disciplinary matter.
- **Ask if you don't understand.** You can't be expected to absorb everything on first explanation. On other hand, if you require clarification again and again, this will not go down well. Take notes if required.
- **Be ready to learn from your experience.** This applies not only to skills and knowledge, but also to work processes, management styles and the personalities of the different people you meet.
- **Be aware that your placement report is likely to include details of your conduct, work rate and attention to detail.**
- **Do your best to meet any deadlines.** These may be critical for work in other parts of the organisation.
- **Keep a diary.** This will be useful when compiling a report and reflecting on your experiences. You should note meetings with supervisors, tasks given and completed, skills learned and your feelings about being in a workplace.
- **Be prepared to carry out menial or tedious tasks.** Even though your ambition may be to direct activities or manage staff yourself, it is important to 'start at the bottom' so you gain a better understanding of procedures and the situation of 'shop-floor' employees.
- **Be aware of job opportunities.** These may be in the company or organisation you are working in, but also in related industries. Think about the organisational structure, where you might fit into this on graduation, what skills you have developed, and where your ambitions might lie.
- **Note that the organisation you are working for may also be interested in your views** – be ready to share both positive and negative feelings and ideas, but always highlight the former.

Sources for further study

Luck, M. (1999) *Your Student Research Project*. Gower, London.

Marshall, P. (1997) *Research Methods: How to Design and Conduct a Successful Project*. How To Books, Plymouth.

McMillan, K.M. and Weyers, J.D.B. (2014) *How to Complete a Successful Research Project*. Pearson Education London.

[For placements, consult the relevant handbook for your university and/or placement provider.]

STUDY EXERCISES

Note: These exercises assume that you have started a research project, or are about to start one, as part of your studies.

32.1 Prepare a project plan. Make a formal plan for your research project, incorporating any milestones dictated by your department, such as interim reports and final submission dates. Discuss your plan with your supervisor and incorporate his or her comments. Refer back to the plan frequently during your project, to see how well you are meeting your deadlines.

32.2 Resolve to write up your work as you go along. Each time you complete an experiment or observation,

write up the materials and methods, analyse the data and draw up the graphs as soon as you can. While you may reject or modify some of these drafts at a later stage, this approach will spread out the majority of the effort and allow time for critical thinking close to the final submission date.

32.3 Devise a computer database for keeping details of your references. Keeping these records up to date will save you a lot of time when writing up. You will need to decide on an appropriate referencing format, or find out about the one followed by your department (see Chapter 5).

Answers to these study exercises are available at go.pearson.com/uk/he/resources



Working with microbes, cells and tissues

33. Learning sterile technique	235
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33 Learning sterile technique

Achieving sterile conditions – you should assume that *all* items of laboratory equipment have contaminating microbes on their surfaces, unless they have been destroyed by some form of sterilisation. These items will only remain sterile if they do not come into contact with the (non-sterile) environment. Similarly, the air in the laboratory is a source of microbes – minimise exposure of cultures to air by imagining an invisible ‘microbial rain’ falling into any open vessel or exposed surface, replacing caps/lids as soon as possible.

Taking account of causes of contamination – these include:

- leaving tubes/plates exposed to air for longer than is necessary
- accidentally touching sterile items against clothing, hair or skin
- putting a sterile instrument (e.g. loop) on the bench and forgetting to resterilise before use
- touching the inside of a cap or Petri plate when handling.



Fig. 33.1 International symbol for a biohazard. Usually red or black on a yellow background, or black on a red background.

Sterile technique (aseptic technique) is the name given to the procedures used in microbiology and cell culture. Although the same general principles apply to all cell types, you are most likely to learn the basic procedures using non-pathogenic bacteria.

Sterile technique serves two main purposes:

1. **To prevent accidental contamination of laboratory cultures** due to microbes from external sources, for example, skin, clothing or the surrounding environment.
2. **To prevent microbial contamination of laboratory workers**, in this instance you and your fellow students.

KEY POINT All cell cultures should be treated as if they contained potentially harmful organisms. Sterile technique forms an important part of safety procedures, and must be followed whenever cell cultures are handled in the laboratory. Avoid any ‘hand-to-mouth’ operations at all times.

Care is required because:

- **you may accidentally isolate a harmful microbe** as a contaminant when culturing a relatively harmless strain
- **some individuals are more susceptible to infection and disease than others** – not everyone exposed to a particular microbe will become ill
- **laboratory culture involves purifying and growing large numbers of microbial cells** – this represents a greater risk than small numbers of the original microbe
- **a microbe may change its characteristics**, perhaps as a result of gene exchange or mutation.

The international biohazard symbol, shown in Fig. 33.1, is used to indicate a significant risk due to a pathogenic microbe (p. 269).

Sterilising equipment and containers

Given the ubiquity of microbes, the only way to achieve sterile conditions is by their destruction or removal. Several methods can be used to achieve this objective:

Heat treatment

This is the most widespread form of sterilisation and is used in several basic laboratory procedures, including:

- **Red-heat sterilisation.** Achieved by heating metal inoculating loops and needles, forceps, needles, etc. in the hottest part of a Bunsen flame (Fig. 33.2). This is a simple and effective form of sterilisation as no microbe will survive even a brief exposure to a naked flame. Flame sterilisation using alcohol is used for glass rods and spreaders.
- **Dry-heat sterilisation.** Here, a hot-air oven is used at a temperature of at least 160 °C for at least 2 h. This method is used for the routine

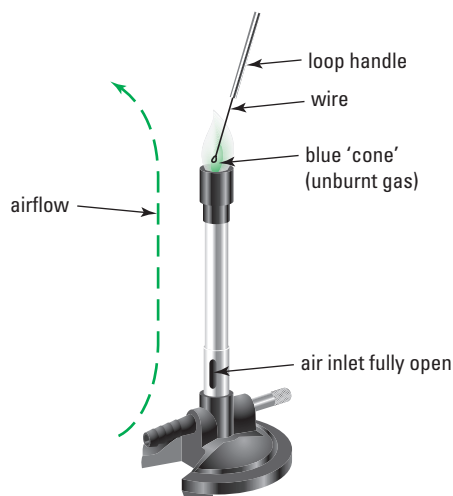


Fig. 33.2 'Flaming' a wire loop. Keep the loop in the hottest part of the Bunsen flame (just outside the blue 'cone') until the wire is red-hot. The convection current created by the heat from the flame (arrowed) creates an area near to the Bunsen where updraught airflow acts to minimise the chance of airborne contamination of items kept within this area.

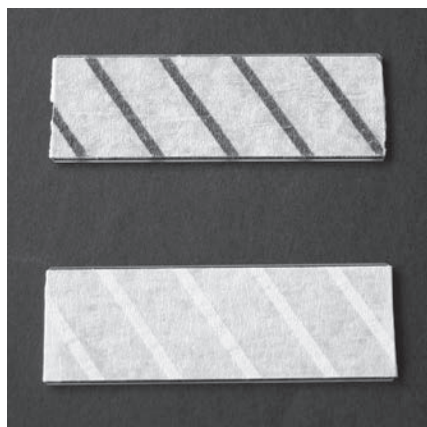


Fig. 33.3 Autoclave tape – the bottom sample is untreated while the upper sample (with dark diagonal lines) has been autoclaved.

Using a sterile filter – most filters are supplied as pre-sterilised items. Make sure you follow a procedure that does not contaminate the filter on removal from its protective wrapping. Handle filters with pre-sterilised forceps (if using red heat sterilization, always let them cool down before use, or you will melt the filter).

sterilisation of laboratory glassware. Dry-heat procedures are of little value for items requiring repeated sterilisation during use.

- **Moist-heat sterilisation.** This is the method of choice for many laboratory items, including most fluids, apart from heat-sensitive media. It is also used to decontaminate liquid media and glassware after use. The laboratory autoclave is used for these purposes. Typically, most items will be sterile after 15 min at 121 °C, although large items may require a longer period. The rapid killing action results from the latent heat of condensation of the pressurised steam, released on contact with cool materials in the autoclave. Although special heat-sensitive tape (Fig. 33.3) is sometimes used to check that the autoclave is operating correctly, a better approach is to use spores of *Bacillus stearothermophilus*.

Radiation

Many disposable plastic items used in microbiology and cell biology are sterilised by exposure to UV or ionising radiation. They are supplied commercially in sterile packages, ready for use. Ultraviolet radiation has limited use in the laboratory (for example, within laminar-flow cabinets and biosafety cabinets), while ionising radiation (for example, γ -rays) requires industrial facilities and cannot be operated on a laboratory scale.

Filtration

Heat-labile solutions (for example, complex macromolecules, including proteins, antibiotics, serum) are particularly suited to this form of sterilisation. The filters come in a variety of shapes, sizes and materials, usually with a pore size of either 0.2 μm or 0.45 μm (see Fig. 21.9). The filtration apparatus and associated equipment is usually sterilised by autoclaving, or by dry heat. Passage of liquid through a sterile filter of pore size 0.2 μm into a sterile vessel is usually sufficient to remove bacteria but not viruses, so filtered liquids are not necessarily virus-free.

Chemical agents

These are usually termed disinfectants, or biocides, and are most often used for the disposal of contaminated items following laboratory use, for example, glass slides and pipettes. They are also used to treat spillages. The term 'disinfection' implies destruction of disease-causing bacterial cells, although spores and viruses may not always be destroyed. Remember that disinfectants require time to exert their killing effect – any spillage should be covered with an appropriate disinfectant and left for at least 10 min before mopping up.

KEY POINT When working with disinfectants and biocides, take care to avoid skin contact or ingestion, as most are toxic and irritant. If you spill disinfectant or biocide on your skin, rinse with plenty of water. If ingested, seek immediate medical help.

Using laboratory equipment

Working area

One of the most important aspects of good sterile technique is to keep your working area as clean and tidy as possible. Start by clearing all items from your working surface, wipe the bench down with disinfectant and then arrange the items you need for a particular procedure so that they are close at hand, leaving a clear working space in the centre of your bench.

Definition

Agar – a jelly-like extract from red algae, consisting of the polysaccharide agarose (at about 70%) and a mix of various oligosaccharides called agaropectins (roughly 30%). It is named from the Malay word meaning 'jelly' (originally agar-agar).

Using molten agar – a water bath (at 45–50 °C) can be used to keep an agar-based medium in its molten state after autoclaving. Always dry the outside of the container on removal from the water bath, to reduce the risk of contamination from microbes in the water, e.g. during pour plating (p. 246).

Working with plastic disposable loops and needles – these are used in many research laboratories: pre-sterilised and suitable for single use, they avoid the hazards of naked flames and the risk of aerosol formation during heating. Do not touch the loop/needle on the outside of the packet during removal. Discard into a disinfectant solution after use.

Using a Bunsen burner to reduce airborne contamination – working close to the convection airflow (updraught) created by a Bunsen flame (Fig. 33.2) reduces the likelihood of particles falling from the air into an open vessel.

Media

Cells may be cultured in either a liquid medium (broth), or a solidified medium (p. 246). The gelling agent used in most solidified media is agar, a complex polysaccharide from marine red algae that produces a stiff transparent gel when used at 1–2% (w/v). Agar is used because it is relatively resistant to degradation by most bacteria and because of its rheological properties – an agar medium melts at 98 °C, remaining solid at all temperatures below this point, enabling it to be used for routine laboratory culture. Once melted however, it does not solidify again until the temperature falls to about 44 °C. This means that heat-sensitive constituents (vitamins, blood, cells, etc.) can be added aseptically to the medium after autoclaving, once the medium has cooled sufficiently (typically, to 50 °C).

Inoculating loops and needles

The initial isolation and subsequent transfer of microbes between containers can be achieved by using a sterile inoculating loop. A sterile inoculating needle ('wire') is most commonly used to transfer broth cultures or agar stab cultures (p. 241). Most teaching laboratories use nichrome wire loops in a metal handle. A nichrome wire loop can be repeatedly sterilised by heating the wire, loop downwards and almost vertical, in the hottest part of a Bunsen flame until the whole wire becomes red-hot. Then the loop is removed from the flame to minimise heat transfer to the handle. After cooling for around 10 s (without touching any other object), it is ready for use. A similar approach is used with nichrome wire needles. Sterile plastic loops and needles are an alternative.

When re-sterilising a contaminated wire loop or needle in a Bunsen flame after use, do not heat the loop too rapidly, as the sample may spatter, creating an aerosol: it is better to soak the loop or needle for a few minutes in disinfectant than to risk heating a fully charged (contaminated) inoculating loop.

Containers

A range of containers are used, including glass test tubes with metal or plastic caps (to prevent airborne contamination) and glass screw-top bottles in a range of sizes (bijou 5–7 mL, McCartney 25–28 mL, medical flat 100–200 mL). There is a risk of contamination of the interior whenever a sterile bottle, flask or test tube is opened. One method that reduces the chance of airborne contamination is quickly to pass the open mouth of the glass vessel through a Bunsen flame. This destroys any microbes on the outer surfaces nearest to the mouth of the vessel. In addition, by heating the air within the neck of the vessel, an outwardly directed air flow is established, reducing the likelihood of microbial contamination.

You should flame the mouth of each vessel immediately after opening and then repeat the procedure just before replacing the top. Caps, lids and cotton wool plugs must not be placed on the bench during flaming and sampling: they should be removed and held using the smallest finger of one hand (Fig. 38.5(a)), to minimise the risk of contamination. This also leaves your remaining fingers free to carry out other manipulations. With practice, you will be able to remove the tops from two tubes, flame each tube and transfer material from one to the other while holding one top in each hand.

For agar-based media in Petri plates (Petri dishes), working close to a lit Bunsen helps reduce the risk of airborne contamination when the

Table 33.1 Classification of microbes on the basis of hazard. The following categories are recommended by the UK Advisory Committee on Dangerous Pathogens (ACDP). A similar approach is followed by the World Health Organization, with four risk groups broadly equivalent to those shown below

Hazard group	Comments
1	Unlikely to cause human disease; generally regarded as safe
2	May cause disease: possible hazard to laboratory workers, minimal hazard to community
3	May cause severe disease: may be a serious hazard to laboratory workers, may spread to community
4	Causes severe disease: is a serious hazard to laboratory workers, high risk to community

lid is removed, due to the upward convection airflow (Fig. 33.2). Work systematically and without undue delay, to minimise the exposure time of the surface of the agar medium to air.

Laminar flow cabinets

These are designed to prevent airborne contamination, for example, when preparing media or subculturing microbes or tissue cultures. Sterile air is produced by passage through a high-efficiency particulate air (HEPA) filter: this is then directed over the working area, either horizontally (towards the operator) or downwards. The operator handles specimens, media, etc., through an opening at the front of the cabinet (Fig. 33.4). Note that standard laminar-flow cabinets do *not* protect the worker from contamination and must not be used with pathogenic microbes: special dedicated biosafety cabinets are used for work with ACDP hazard group 3 and 4 microbes (Table 33.1) and for samples that might contain such pathogens.

Minimising infection risk through safe working practices

The following steps will reduce the chance of aerosol formation:

- use stoppered tubes when shaking, centrifuging or mixing microbial suspensions
- pour solutions gently, keeping the difference in height to a minimum
- discharge pipettes on to the side of the container.



Fig. 33.4 A laminar flow cabinet. Air is drawn in and through a HEPA filter in either the back or the top, exiting the front through the opening created by the moveable glass door. When in use, raise door only enough to enable you to work on the workbench.

Using glass pipettes – these are typically plugged with cotton wool at the top before being autoclaved inside a metal can. Flame the open end of the can on removal of a pipette, to prevent contamination of the remaining pipettes. Autopipettors and sterile disposable tips (pp. 143–4) offer an alternative approach.

SAFETY NOTE The most obvious hazards when handling microbial cultures are those due to ingestion or entry via a cut in the skin – all cuts should be covered with a plaster or disposable plastic gloves. A less obvious source of hazard is the formation of aerosols of liquid droplets from microbial suspensions, with the risk of inhalation, or surface contamination of other objects.

Other general rules that apply in microbiology laboratories include:

- **take care with sharp instruments, including needles and glass Pasteur pipettes**
- **avoid all ‘hand-to-mouth’ actions**, for example, chewing the end of your pencil while thinking
- **do not pour waste cultures down the sink** – they must be autoclaved or treated with disinfectant
- **put other contaminated items (for example, slides, pipettes) into disinfectant after use**
- **wipe down your bench with disinfectant when practical work is complete**
- **always wash your hands thoroughly before leaving the laboratory (Fig. 33.5).**

KEY POINT Remember that microscopic droplets below 5 μm can remain suspended in air for extended periods, due to microturbulence; they can also penetrate into the alveoli of the lungs, creating a greater risk of infection, as seen in Legionnaires’ disease and COVID-19.

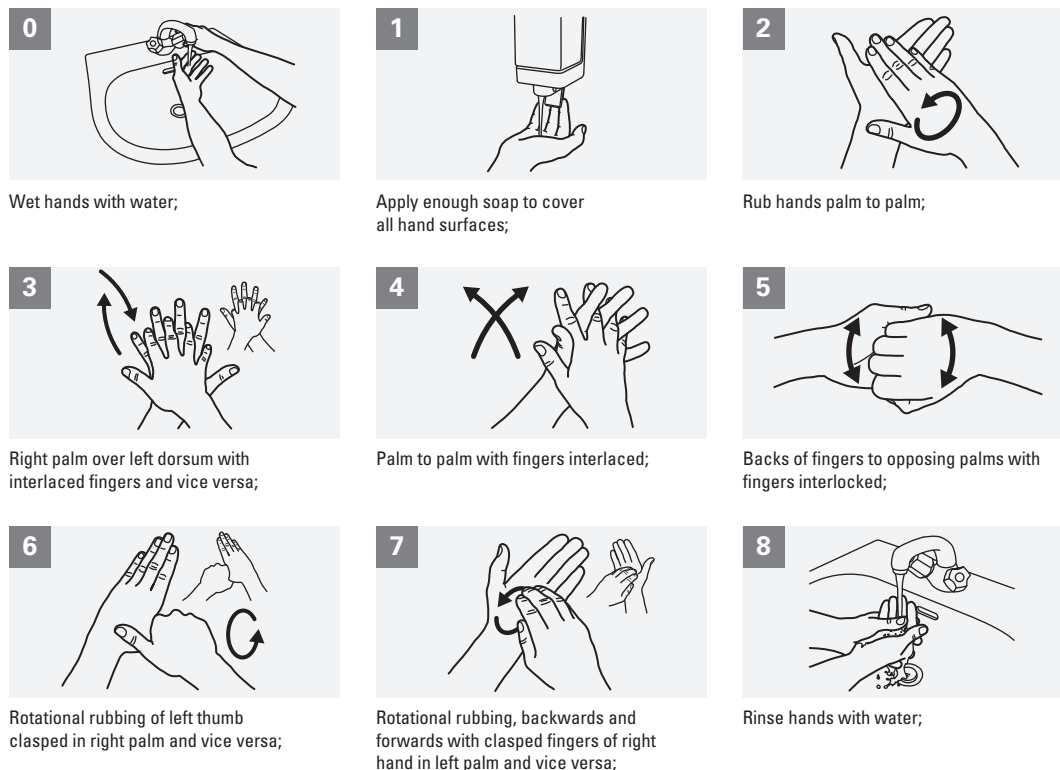


Fig. 33.5 Hand washing, based on World Health Organisation guidelines (duration 40–60 s). (0) wet hands with water (1) apply soap, then rub: (2) palm-to-palm (3) palm-to-back with interlaced fingers (for both palms/back) (4) palm-to-palm with interlaced fingers (5) interlocked fingers (6) thumbs (7) both palms, and then (8) rinse with water. Then, dry hands with paper towel and use to turn off tap, or use elbow-operated tap.

Labelling Petri plates and culture tubes – record the following information on the *base* of each plate, or on the side of each tube:

- date
- the growth medium (in abbreviated format, if appropriate)
- your name or initials
- brief details of the experimental treatment.

Labelling plates and cultures

Petri plates should always be labelled on the *base*, rather than the lid, using a permanent (spirit-based) marker. Restrict your labelling to the outermost region of the plate, to avoid problems when counting colonies, assessing growth, etc. After labelling, incubate Petri plates upside down (to avoid problems due to condensation of water droplets on the lid) in a temperature-controlled incubator (often at 37 °C) for an appropriate period (usually 18–72 h). Plates are also usually kept upside down (agar uppermost) on the lab bench – following incubation, the base (containing medium and microbes) can then be lifted from the lid and examined, then replaced. Culture tubes need similar labelling, and are usually stored and incubated in a rack.

Sources for further study

Anon. (1995) *Advisory Committee on Dangerous Pathogens: Categorisation of Biological Agents According to Hazard and Categories of Containment*, 4th edn. HSE Books, London.

Anon. (2013) *Advisory Committee on Dangerous Pathogens: Approved List of Biological Agents*. Available: <https://www.hse.gov.uk/pubns/misc208.pdf> Last accessed 06/05/21.

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Hawkey, P. and Lewis, D. (2004) *Medical Bacteriology: A Practical Approach*, 2nd edn. Oxford University Press, Oxford.

World Health Organization (2004) *Laboratory Biosafety Manual*, 3rd edn. Available: <https://www.who.int/csr/resources/publications/biosafety/en/Biosafety7.pdf> Last accessed 06/05/21.

STUDY EXERCISES

33.1 Decide on the best method of sterilisation. What would be the most appropriate method of sterilisation for the following items?

- a box of 100 plastic tips to be used with a pipettor
- a 50 mL batch of blood, for use in 5% v/v blood agar plates
- a 1 L batch of MacConkey agar
- ten 5 mL glass pipettes
- microbiological wire, used for 'stab' cultures
- a 10 mL sample of a heat-sensitive solution of an antibiotic, to be used as a component of a selective isolation medium.

33.3 Find out the biohazard classification (UK ACDP categorisation – Table 33.1) for the following microbes:

- Salmonella typhimurium*
- Leptospira interrogans*
- Shigella dysenteriae* (type 1)
- Escherichia coli* K12
- Escherichia coli* O157
- Human immunodeficiency virus (HIV)
- Cryptococcus neoformans* var. *neoformans*
- Mycobacterium tuberculosis*
- Lactobacillus plantarum*
- Marburg virus.

33.3 Write out the steps involved in transferring a droplet of cell culture from a tube of broth culture to a fresh tube of sterile broth.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

34 Culturing cells and measuring growth

Definitions

Chemoautotroph – an organism that acquires energy from the oxidation of simple inorganic compounds, fixing CO_2 as a source of carbon (chemosynthetic metabolism).

Heterotroph – an organism that uses complex organic carbon compounds as a source of carbon and energy.

Photoautotroph – an organism that uses light as a source of energy and CO_2 as a carbon source (photosynthetic metabolism).

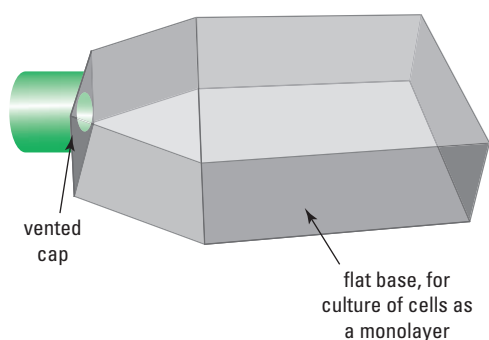


Fig. 34.1 Plastic flask for animal cell culture – this design provides a large surface area for growth of an adherent monolayer of cells.

Harvesting bacteria or yeast from an agar plate – colonies can be harvested using a sterile loop, providing large numbers of cells without the need for centrifugation. The cells are relatively free from components of the growth medium; this is useful if the medium contains substances that interfere with subsequent procedures.

Subculturing – when subculturing microbes from a colony on an agar medium, take your sample from the growing edge, so that viable cells are transferred.

Microbial, animal and plant cell culture methods are based on the same general principles, requiring:

- a **pure culture** (also known as an axenic culture), perhaps isolated as part of an earlier procedure (p. 242), or from a culture collection
- a **suitable nutrient medium** to provide the necessary components for growth; this medium must be sterilised before use
- **satisfactory growth conditions** including temperature, pH, atmospheric requirements, ionic and osmotic conditions
- **sterile technique** (Chapter 33) to maintain the culture in pure form.

Heterotrophic animal cells, fungi and many bacteria require appropriate organic compounds as sources of carbon and energy. Non-exacting bacteria can use a wide range of compounds and they are often grown in media containing complex natural substances (including meat extract, yeast extract, soil, blood). Fungi are mostly grown in slightly acidic, complex media. Animal cells have more stringent growth requirements (pp. 296–7).

Photoautotrophic bacteria, cyanobacteria and algae are grown in a mineral medium containing inorganic ions including chelated iron, with a light source and CO_2 supply. Plant cells may require additional vitamins and hormones (p. 296). For chemoautotrophic bacteria, the light source is replaced by a suitable inorganic energy source, for example, H_2S for sulfur-oxidising bacteria, $\text{NH}_3/\text{NH}_4^+$ for nitrifying bacteria, etc.

Growing cells on solidified media

Many cellular microbes can be cultured on agar-based medium (p. 237).

Animal cells are often grown as an adherent monolayer on the surface of a plastic or glass culture vessel (Fig. 34.1), rather than on an agar-based medium.

Several types of culture vessel are used:

- **Petri plates (Petri dishes)**: usually the pre-sterilised, disposable plastic type, providing a large surface area for growth.
- **Glass bottles or test tubes**: these provide sufficient depth of agar medium for prolonged growth of bacterial and fungal cultures, avoiding problems of dehydration and salt crystallisation. Inoculate aerobes on the surface and anaerobes by stabbing down the centre, into the base (stab culture).
- **Flat-sided bottles**: these are used for animal cell culture, to provide an increased surface area for attachment and allow growth of cells as a surface monolayer. Usually plastic and disposable.

The dynamics of growth are usually studied in liquid culture, apart from certain rapidly growing filamentous fungi (moulds, pp. 276–7), where increases in diameter of the growing mycelium can be measured accurately, for example, using Vernier calipers.

KEY POINT An important benefit of agar-based culture systems is that an individual cell inoculated onto the surface can develop to form a visible colony that can then be manipulated or further cultured in the laboratory: this is the basis of most microbial isolation and purification methods, including the streak dilution, spread-plate and pour-plate procedures used for bacteria and yeast (pp. 273–4).

Growing cells in liquid media

Many cells, apart from primary cultures of animal cells, can be grown as a homogeneous unicellular suspension in a suitable liquid medium (broth), where growth is usually considered in terms of cell *number* (population growth) rather than cell size. Most liquid culture systems need agitation, to ensure adequate mixing and to keep the cells in suspension. An Erlenmeyer flask of 100–2000 mL capacity (Fig. 34.2) can be used to grow a batch culture on an orbital shaker, operating at 20–250 cycles per minute. For aerobic organisms, the surface area of such a culture should be as large as possible: you should use a volume of medium not more than 20% of the flask volume.

Larger cultures may need to be aerated using sterile air and mixed using a magnetic stirrer rather than an orbital shaker. The simplest method of air sterilisation is filtration, using glass wool, non-absorbent cotton wool or a commercial filter unit of appropriate pore size (usually 0.2 μm). Air is introduced via a sparger (a glass tube with many small holes at the end, so that small bubbles are produced) near the bottom of the culture vessel to increase the surface area and enhance gas exchange. More complex systems have baffles and paddles to further improve mixing and gas exchange.

Liquid culture systems may be subdivided under two broad headings: batch culture and continuous culture.

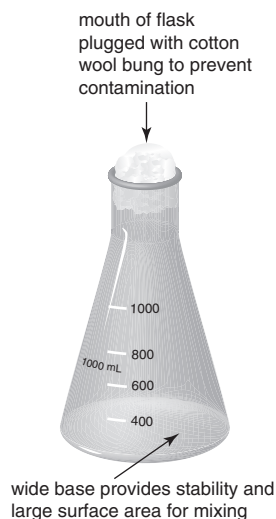


Fig. 34.2 Conical (Erlenmeyer) flask.

Batch culture

This is the most common approach for routine liquid culture. Cells are inoculated into a sterile vessel containing a fixed amount of growth medium. Your choice of vessel will depend upon the volume of culture required: larger-scale vessels (for example, 1 L and above) are often called ‘fermenters’ or ‘bioreactors’, particularly in biotechnology. Growth within the vessel usually follows a predictable S-shaped (sinusoidal) curve when plotted in log–linear format (Fig. 34.3), divided into four components:

- 1. Lag phase:** the initial period when no increase in cell number is seen. The larger the inoculum of active cells the shorter the lag phase will be, provided the cells are transferred from similar growth conditions.
- 2. Log phase, or exponential phase:** where cells are growing at their maximum rate. This may be quantified by the growth rate constant, or specific growth rate (μ , or k), where:

$$\mu = \frac{2.303 (\log N_x - \log N_0)}{(t_x - t_0)} \quad [34.1]$$

where N_0 is the initial number of cells at time t_0 and N_x is the number of cells at time t_x . For times specified in hours, μ is expressed as h^{-1} .

Prokaryotes grow by binary fission, whereas eukaryotes grow by mitotic cell division; in both cases each cell divides to give two identical

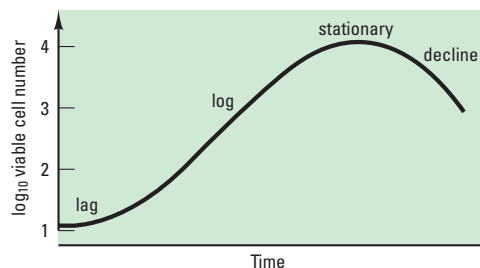


Fig. 34.3 Population growth curve for cells in batch culture (liquid medium).

Example Suppose you counted 2×10^3 cells ($\log_{10} = 3.30$) per unit volume at t_0 and 6.3×10^4 cells ($\log_{10} = 4.80$) after 2 h (t_x).

Substitution into eqn [34.1] gives
 $[2.303(4.8 - 3.3)] \div 2 = 1.727 \text{ h}^{-1}$ (or
 0.0288 min^{-1}).

Substituting the same values into eqn [34.2] gives:
 $[0.301 \times 2] \div [4.8 - 3.3] = 0.40 \text{ h}$ (or
 24 min).

Working with logarithms – note that there is no log value for zero, so you cannot plot zero on a log-linear growth curve or on a death curve. Your graph should end at the last measured 'count', or at the lower limit of detection.

Example Suppose you counted 5.2×10^5 cells ($\log_{10} = 5.716$) per unit volume at t_0 and 3.7×10^3 cells ($\log_{10} = 3.568$) after 60 min (t_x). Substitution into eqn [34.3] gives $60 \div [2.148] = 27.9 \text{ min}$. To the nearest minute, this gives a value for d of 28 min.

offspring. Consequently, the doubling time or generation time (g , or T_2) is:

$$g = \frac{0.031(t_x - t_0)}{(\log N_x - \log N_0)} \quad [34.2]$$

Cells grow at different rates, with doubling times ranging from under 20 min for some bacteria to 24 h or more for animal and plant cells. Exponential phase cells are often used in laboratory experiments, since growth and metabolism are nearly uniform.

- 3. Stationary phase:** growth decreases as nutrients are depleted and waste products accumulate. Any increase in cell number is offset by death. This phase is usually termed the 'plateau' in animal cell culture.
- 4. Decline phase, or death phase:** this is the result of prolonged starvation and toxicity, unless the cells are subcultured. Like growth, death often shows an exponential relationship with time, which can be characterised by a rate constant (death rate constant), equivalent to that used to express growth or, more often, as the decimal reduction time (d , or T_{90}), the time required to reduce the population by 90%:

$$d = \frac{t_x - t_0}{\log N_x - \log N_0} \quad [34.3]$$

Some cells undergo rapid autolysis at the end of the stationary period while others show a slower decline.

Batch-culture methods can be used to maintain stocks of particular organisms; cells are subcultured to fresh medium before they enter the decline phase. However, primary cultures of animal cells have a finite life unless transformed to give a continuous cell line, capable of indefinite growth (pp. 296–7).

Continuous culture

This is a method of maintaining cells in exponential growth for an extended period by continuously adding fresh growth medium to a culture vessel of fixed capacity. The new medium replaces nutrients and displaces some of the culture, diluting the remaining cells and allowing further growth.

After inoculating the vessel, the culture is allowed to grow for a short time as a batch culture, until a suitable population size is reached. Then medium is pumped into the vessel: the system is usually set up so that any increase in cell number due to growth will be offset by an equivalent loss due to dilution, i.e. the cell number within the vessel is maintained at a steady state. The cells will be growing at a particular rate (μ), counterbalanced by dilution at an equivalent rate (D):

$$D = \frac{\text{flow rate}}{\text{vessel volume}} \quad [34.4]$$

where D is expressed per unit time (for example, h^{-1}). In a chemostat, the growth rate is limited by the availability of one nutrient in the inflowing medium, usually either a source of carbon or nitrogen (see Fig. 34.4). In a turbidostat, the input of medium is controlled by the turbidity of the

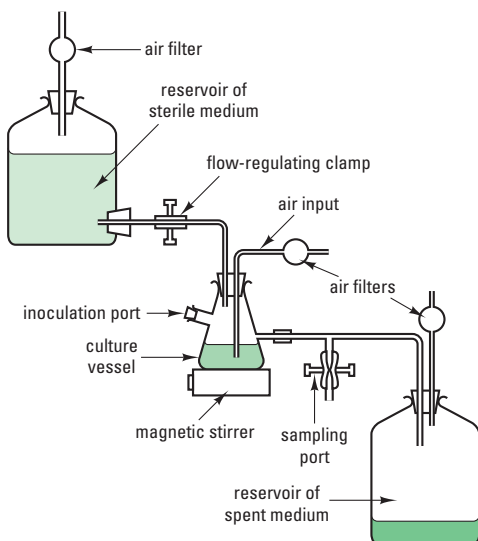


Fig. 34.4 Example of a two-dimensional lab equipment diagram of components of a chemostat.

Example Suppose a continuous culture system of 2000 mL volume had a flow of 600 mL over a period of 40 min (flow rate $600 \div 40 = 15 \text{ mL min}^{-1}$). Substitution into eqn [34.4] gives a dilution rate D of $15 \div 2000 = 0.00075 \text{ min}^{-1}$ or $0.00075 \times 60 = 0.45 \text{ h}^{-1}$.

Example Suppose you wanted to convert a doubling time of 20 min to a specific growth rate. Rearrangement of eqn [34.5] gives $\mu = 0.693 \div 20 = 0.03465 \text{ min}^{-1}$ ($= 2.08 \text{ h}^{-1}$). For the example given on page 243, with a growth rate of 0.45 h^{-1} , substitution into eqn [34.4] gives $0.693/0.45 = 1.54 \text{ h}$ (approx. 92 min).

culture, measured using a photocell. A turbidostat is more complex than a chemostat, with additional equipment and controls.

To determine the specific growth rate (μ , or k) of a continuous culture:

1. **Measure the flow of medium through the vessel over a known time interval** (for example, connect a sterile measuring cylinder or similar volumetric device to the outlet), to calculate the flow rate.
2. **Divide the flow rate by the vessel volume** (eqn [34.4]) to give the dilution rate (D).
3. **This equals the specific growth rate**, since $D = \mu$ at steady state.
4. **If you want to know the doubling time (g), calculate using the relationship:**

$$g = \frac{0.693}{\mu} \quad [34.5]$$

(Note that eqn [34.5] also applies to exponential phase cells in batch culture and is useful for interconverting g and μ .)

Continuous culture systems are more complex to set up and maintain, when compared to batch cultures. They are prone to contamination, having additional vessels for fresh medium and waste culture: strict aseptic technique is necessary when the medium reservoir is replaced, and during sampling and harvesting. However, they offer several advantages over batch cultures, including:

- **The physiological state of the cells is more clearly defined**, since actively growing cells at the same stage of growth are provided over an extended time period. This is useful for biochemical and physiological studies.
- **Monitoring and control can be automated.**
- **Modelling can be carried out for biotechnology/fermentation technology.**

Measuring growth in cell cultures

The most widely used methods of measuring growth are based on cell number.

Direct microscopic counts

One of the simplest methods is to count the cells in a known volume of medium using a microscope and a counting chamber or haemocytometer (Box 34.1). While this gives a rapid assessment of the total cell number, it does not discriminate between living and dead cells. It is also time-consuming as a large number of cells must be counted for accurate measurement. Also, it may be difficult to distinguish individual cells, for example, for cells growing in clumps.

Electronic particle counters

These instruments can be used to give a direct (total) count of a suspension of microbial cells. For example, the Coulter counter detects particles due to change in electrical resistance when they pass through a small aperture in a glass tube filled with an electrolyte (Fig. 34.5). It gives a rapid count based on a larger number of cells than direct microscopy. It is well suited for repeat measurements or large sample numbers and can be linked to a computer for data processing. If correctly calibrated, the Coulter counter

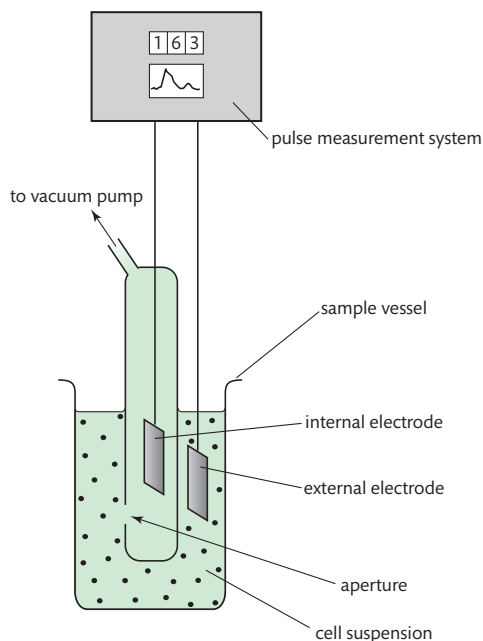


Fig. 34.5 Components of an electronic particle counter. During operation, the cell suspension is drawn through the aperture by the vacuum, creating a 'pulse' of resistance between the two electrodes as each cell passes through the aperture.

Box 34.1 How to use a counting chamber or haemocytometer

A counting chamber is a specially designed slide containing a chamber of known depth with a grid etched on to its lower surface. When a flat coverslip is placed over the chamber, the depth is uniform. Use as follows:

- 1. Place the special coverslip over the chamber.** Press the edges firmly, to ensure that the coverslip makes contact with the surface of the slide, but take care that you do not break the slide or coverslip by using too much force. When correctly positioned, you should be able to see interference rings (Newton's rings) at the edge of the coverslip.
- 2. Add a small amount of your cell suspension to fill the central space above the grid.** Place on the microscope stage and allow the cells to settle (2–3 min).
- 3. Examine the grid microscopically.** Using the $\times 10$ objective lens first, since the counting chamber is far thicker than a standard microscope slide. Then switch to the $\times 40$ objective: take care not to scratch the surface of the objective lens, as the special coverslip is thicker than a normal coverslip. For a dense culture, the small squares are used, while the larger squares are used for dilute suspensions. You may need to dilute your suspension if it contains more than 30 cells per small square.
- 4. Count the number of cells in several squares:** at least 600 cells should be counted for accurate measurements. Include those cells that cross the upper and left-hand boundaries, but not those that cross the lower or right-hand rulings. A hand tally may be used to aid counting. Motile cells must be immobilised prior to counting (e.g. by killing with a suitable biocide).
- 5. Divide the total number of cells (C) by the number of squares counted (S),** to give the mean cell count per square.
- 6. Determine the volume (in mL) of liquid corresponding to a single square (V),** e.g. a Petroff-Hausser chamber has small squares of linear dimension 0.2 mm, giving an area of 0.04 mm^2 ; since the depth of the chamber is 0.02 mm, the volume is $0.04 \times 0.02 = 0.0008 \text{ mm}^3$; as there are 1000 mm^3 in 1 mL, the volume of a small square is $8 \times 10^{-7} \text{ mL}$; similarly, the volume of a large square (equal to 25 small squares) is $2 \times 10^{-5} \text{ mL}$. Note that other types of counting chamber will have different volumes: check the manufacturer's instructions. For example, the improved Neubauer chamber (Fig. 34.6) has small squares of volume $0.00025 \text{ mm}^3 = 2.5 \times 10^{-7} \text{ mL}$.
- 7. Calculate the cell number per mL by dividing the mean cell count per square by the volume of a single square (in mL).**

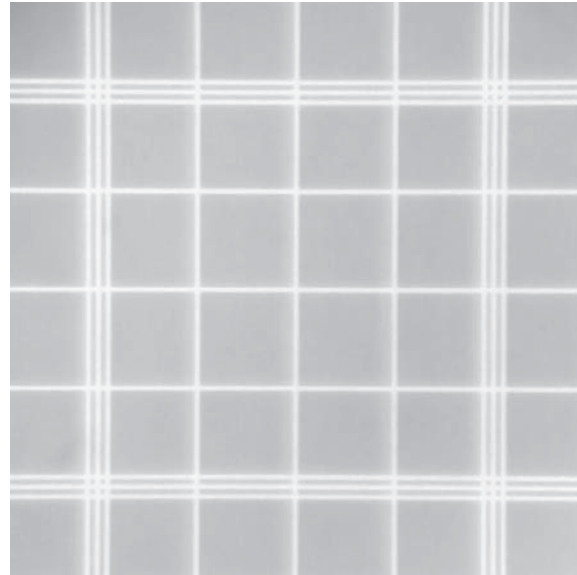


Fig. 34.6 Haemocytometer grid (improved Neubauer rulings) viewed microscopically. The large square (delimited by triple etched lines) has a volume of $1/250 \text{th mm}^3$ ($0.04 \text{ mm}^3 = 4 \text{ nL}$) while each small square (16 contained within the large square) has a volume of $1/4000 \text{ mm}^3$ ($0.00025 \text{ mm}^3 = 0.25 \text{ nL}$). Note that the boundary line for squares delimited by triple-etched lines is the *middle* line, so this line must be used when counting (see Fig. 28.4, for how to deal with objects straddling the gridlines).

- 8. Remember to take account of any dilution of your original suspension** in your final calculation by multiplying by the reciprocal of the dilution (M), e.g. if you counted a 1 in 20 dilution of your sample, multiply by 20, or if you diluted to 10^{-5} , multiply by 10^5 .

The complete equation for calculating the total microscopic count is:

$$\text{Total cell count (per mL)} = (C \div S \div V) \times M \quad [34.6]$$

For example, if the mean cell count for a hundred-fold dilution of a cell suspension, counted using a Petroff-Hausser chamber, was 12.4 cells in ten small squares, the total count would be

$$(12.4 \div 10 \div 8 \times 10^{-7}) \times 10^2 = 1.55 \times 10^8 \text{ mL}^{-1}$$

A simpler, less accurate approach is to use a known volume of sample under a coverslip of known area on a standard glass slide, counting the number of cells per field of view using a calibrated microscope of known field diameter, then multiplying up to give the cell number per mL (see p. 247).

Using alternative approaches for measuring growth – these include biomass, dry weight, turbidity (light scattering: nephelometry), uptake of radiolabelled substrates, absorbance or assay of any major cellular component, e.g. protein, nucleic acid, ATP, etc.

Using culture-based methods and selective media – injured cells and stressed microbes may not be able to grow on selective media under standard aerobic conditions unless a non-selective resuscitation stage is added. Alternative approaches involve the addition of scavengers of reactive oxygen species (ROS), e.g. pyruvate and/or catalase.

Definition

CFU – colony-forming unit: a cell or group of cells giving rise to a single colony on a solidified medium.

Using alternative approaches in plate counting – when large numbers of samples have to be counted, a single Petri plate of agar-based medium can be divided into segments and a single droplet of each dilution placed into the appropriate segment, enabling a dilution series to be contained within a single plate ('Miles and Misra' droplet counting).

Adopting molecular approaches to enumerating microbes – DNA-based methods can be used, including real-time quantitative polymerase chain reaction (RT-qPCR, Chapter 68).

can also measure cell sizes. A major limitation of electronic counters is their lack of discrimination between living cells, dead cells, cell clumps and other particles (for example, dust). In addition, the instrument must be set up and calibrated by trained personnel. Flow cytometry is a more specialised alternative, since particles can be sorted as well as counted (see Givan, 2013).

Culture-based counting methods

A variety of culture-based techniques can be used to determine the number of cellular microbes in a sample. A major assumption of such methods is that, under suitable conditions, an individual viable microbial cell will be able to multiply and grow to give a visible change in the growth medium, for example, a colony on an agar-based medium, or turbidity ('cloudiness') in a liquid medium. You are most likely to gain practical experience using bacterial cultures, counted by one or more of the following methods:

- **Spread- or pour-plate methods** ('plate counts', p. 247). The most widespread approach is to transfer a suitable amount of a known dilution of the sample to an agar medium, incubate under appropriate conditions and then count the resulting colonies (Box 34.2).
- **Membrane filtration**. For microbial cell suspensions where the expected cell number is lower than 10 CFU mL^{-1} , pass the sample through a sterile filter (pore size $0.2 \mu\text{m}$ or $0.45 \mu\text{m}$). Then incubate the filter on a suitable medium until colonies are produced, giving a count by dividing the mean colony count per filter by the volume of sample filtered.
- **Multiple tube count**, or most probable number (MPN). A microbiological technique where the sample is diluted and known volumes are then transferred to several tubes of liquid medium (typically, five tubes at three volumes), chosen so that there is a low probability of the smallest volumes containing a viable cell. After incubation, the number of tubes showing growth (turbidity) is compared to tabulated values to give the most probable number (MPN per mL).

The principal advantage of culture-based counting procedures is that dead cells will not be counted. However, for such techniques, the incubation conditions and media used may not allow growth of all cells, underestimating the true viable count. This is exacerbated for stressed or injured cells, which may not always grow in standard culture media/conditions. Further problems are caused by cell clumping and dilution errors. In addition, such methods require sterile apparatus and media and the incubation period is lengthy before results are obtained.

An alternative approach is to use direct microscopy, combined with 'vital' or 'mortal' staining. For example, the direct epifluorescence technique (DEFT) uses acridine orange and UV epifluorescence microscopy to separate living and dead bacteria, while neutral red is a vital stain used for plant cells. Chapter 25 gives examples of vital/mortal stains for other cell types. A further approach is to use DNA-based methods, such as the polymerase chain reaction (PCR, Chapter 68), to estimate the number of cells present.

Using bioassays

A bioassay is a method of quantifying a chemical substance (analyte) by measuring its effect on a biological system under controlled conditions. The hypothetical underlying phenomena are summarised by the relationship:



Box 34.2 How to make a plate count of a microbial cell suspension using an agar-based medium

- 1. Prepare serial decimal dilutions of the cell suspension in a sterile diluent (pp. 147–8).** The most widely used diluents are 0.1% w/v peptone water or 0.9% w/v NaCl, buffered at pH 7.3. Take care that you mix each dilution thoroughly (e.g. using a vortex mixer) before making the next one. For soil, food, or other solid samples, make the initial decimal dilution by taking 1 g of sample and making this up to 10 mL using a suitable diluent. Vigorous agitation or homogenisation may be required for organisms growing in clumps. The number of decimal dilutions required for a particular sample will be governed by your expected count: dilute until the expected number of viable cells is around 100–1000 mL⁻¹.
- 2. Transfer an appropriate volume (e.g. 0.05–0.5 mL) of the lowest dilution to an agar plate** using either the spread-plate method or the pour-plate procedure (pp. 273–4). At least two, and preferably more, replicate plates should be prepared for each sample, since the confidence limits for small counts of single samples is large. You may also wish to prepare replicate plates for more than one dilution, if you are unsure of the expected number of viable cells.
- 3. Incubate under suitable conditions for 18–72 h, then count the number of colonies on each replicate plate at the most appropriate dilution.**
- 4. Calculate the colony count per mL of that particular dilution** by dividing by the volume (in mL) of liquid transferred to each plate (V).
- 5. Now calculate the count per mL of the original sample** by multiplying by the reciprocal of the dilution: this is the multiplication factor (M); e.g. for a dilution of 10⁻³, the multiplication factor would be 10³. For soil, food or other solid samples, the count should be expressed per g of sample.

The complete equation for calculating the viable count is:

$$\text{Count per mL (or per g)} = (C \div V) \times M \quad [34.7]$$

For example, for a sample with a mean colony count of 5.5 colonies per plate for a volume of 0.05 mL at a dilution of 10⁻⁷, the count would be:

$$(5.5 \div 0.05) \times 10^7 = 1.1 \times 10^9 \text{ CFU mL}^{-1}$$

The count should be reported as colony-forming units (CFU) per mL, rather than as cells per mL, since a colony may be the product of more than one cell, particularly in filamentous microbes or in organisms with a tendency to aggregate. You should also be aware of the problems associated with counts of zero – these are best recorded as '<1', and you should then apply the appropriate correction factors for dilution and volume to obtain the lower detection limit. For example, a zero count (<1) of 100 µL of a five-fold dilution gives a lower detection limit of (<1 ÷ 0.1) × 5 = <50 CFU mL⁻¹.

The most accurate results will be obtained for plates containing 30–300 colonies. Mark the base of the plate with a spirit-based pen each time you count a colony. Determine the mean colony count per plate at this dilution (C).

where A is the analyte, Rec the receptor, ARec the analyte–receptor complex and R the response. This relation is analogous to the formation of product from an enzyme–substrate complex and, using similar mathematical arguments to those of enzyme kinetics (pp. 463–4), it can be shown that the expected relationship between [A] and rate of response is hyperbolic (sigmoidal in a log–linear plot as shown in Fig. 34.7). This pattern of response is usually observed in practice if a wide enough range of [A] is tested.

To carry out the assay, the response elicited by the unknown sample is compared to the response obtained for differing concentrations of the substance, as shown in Fig. 36.7. When fitting a curve to standard points and estimating unknowns, the available methods you can adopt, in order of increasing accuracy, are:

1. fitting by eye
2. using linear regression on a restricted 'quasi-linear' portion of the assay curve

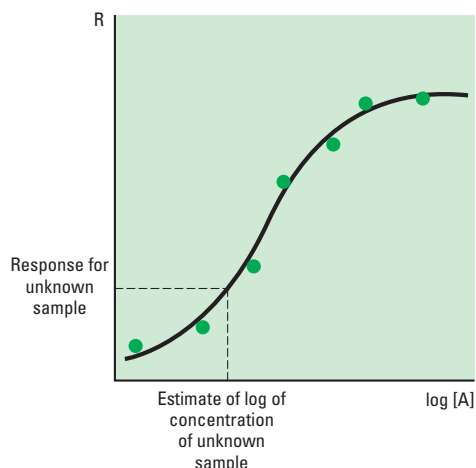


Fig. 34.7 Typical bioassay response curve, showing estimation of an unknown sample. Closed circles represent responses with standard samples. R = response; A = analyte.

Example The following are typical bioassays:

- measuring the amount of a plant growth substance in a plant extract using the coleoptile straight growth assay;
- measuring the effect of a drug on the dilation of a person's pupils;
- measuring the mutagenic properties of a chemical compound in the Ames test (see Box 34.3).

3. linearisation (for example, by probit transformation) followed by regression
4. non-linear regression (for example, to the Morgan–Mercer–Flodin equation).

In general, bioassay techniques have more potential faults than physicochemical assay techniques (Chapter 56). These may include the following:

- **A greater level of variability:** error in the estimate of the unknown compound will result because no two organisms will respond in exactly the same way. Assay curves vary through time, and because they are non-linear, a full standard curve is required each time the assay is carried out.
- **Lack of chemical information:** bioassays provide information about *biological* activity; they say little about the chemical structure of an unknown compound. The presence of a specific compound may need to be confirmed by a physicochemical method (for example, mass spectrometry, Chapter 47).
- **Possibility of interference:** while many bioassays are very specific, it is possible that different chemicals in the extract may influence the results.

Despite these problems, bioassays are still much used. They are ‘low-tech’ and cost-effective for lab classes. They often allow detection at very low concentrations. Bioassays also provide the means to assess the biological activity of chemicals and to study changes in sensitivity to a chemical, which physicochemical techniques cannot do. Changes in sensitivity may be evident in the shape of the dose–response curve and its position on the concentration axis.

Bioassays can involve responses of whole organisms or parts of organisms. ‘Isolated’ responding systems (for example, excised tissues or cells, Chapter 41) decrease the possibility of interference from other parts of the organism. Disadvantages include disruption of nutrition and wound damage during excision. Isolation can continue down to the molecular level, as in immunoassays (Chapter 53).

Bioassays are the basis for characterising the efficacy of drugs and the toxicity of chemicals. Here, response is often treated as a quantal (all-or-nothing) event. The E_{50} is defined as that concentration of a compound causing 50% of the organisms to respond. Where death is the observed response, the LD_{50} describes the concentration of a chemical that would cause 50% of the test organisms to die within a specified period under a specified set of conditions. Box 34.3 presents details of the Ames test, a widely used bioassay used to assess the mutagenicity of chemicals.

Considerations when setting up a bioassay

When thinking about using a bioassay, take account of the following points:

- The response should be easily measured and as metabolically ‘close’ to the initial binding event as possible.
- The experimental conditions should mimic the *in vivo* environment.

Box 34.3 How to test for mutagenicity using the Ames test – an example of a widely used bioassay

Chemical carcinogens can be identified by the formation of tumours in laboratory animals exposed to the compound under controlled conditions. However, such animal bioassays are time-consuming and expensive. Dr Bruce Ames and co-workers have shown that most carcinogens are also mutagens, i.e. they will induce mutational changes in DNA. The Ames test makes use of this correlation to provide a simple, rapid and inexpensive bioassay for the initial screening of potential carcinogens. The test makes use of particular strains of *Salmonella typhimurium* with the following characteristics:

- **histidine auxotrophy** – the tester strains are unable to grow on a minimal medium without added histidine: this characteristic is the result of specific mutational changes to the DNA of these strains, including base substitutions and frame shifts;
- **increased cell envelope permeability**, to permit access of the test compound to the cell interior;
- **defects in excision repair systems and enhanced error-prone repair systems**, to reduce the likelihood of DNA repair after treatment with a potential mutagen.

When grown in the presence of a chemical mutagen, the bacteria may revert to prototrophy as a result of back mutations that restore the wild-type phenotype: such revertants grow independently of external histidine and are able to form colonies on minimal medium, unlike the original test strains. The extent of reversion can be used to assess the mutagenic potential of a particular chemical compound. Since many chemicals must be activated *in vivo*, the test incorporates a rat liver homogenate (so-called 'S-9 activator', containing microsomal enzymes) to simulate the metabolic events within the liver. The tester strains are mixed with S-9 activator and a small amount of molten soft agar, then poured as a thin agar overlay (top agar) on a minimal medium plate. The top agar layer contains a very small amount of histidine, to allow the tester strains to divide a few times and express any mutational changes (i.e. prototrophy).

You can perform the test in one of two ways.

1. **Spot test:** here, you place a concentrated drop of the test compound at the centre of the plate, either directly on the agar surface, or on a small filter paper disc. The test compound will diffuse into the agar and revertants

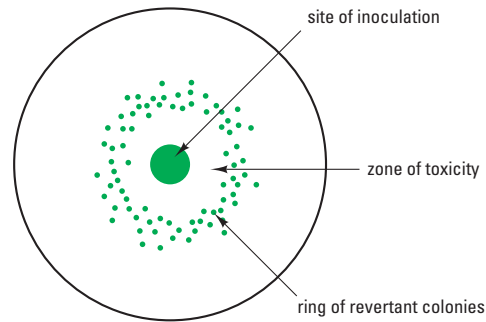


Fig. 34.8 Typical outcome of spot test (Ames test).

appear as a ring of colonies around the site of inoculation, as shown in Fig. 34.8. The distance of the ring of colonies from this site provides a measure of the toxicity of the compound, while the number of colonies within the ring gives an indication of the relative mutagenicity of the test substance. The spot test is often carried out in a simplified form, without added S-9 activator, as a rapid preliminary test prior to quantitative analysis.

2. **Agar incorporation test:** in this method, you mix known amounts of the test compound separately with molten top agar and the other constituents and pour them onto separate minimal agar plates. After incubation for 48 h, revertants will appear as evenly dispersed colonies throughout the agar overlay. The number of colonies reflects the relative mutagenicity of the test compounds, with a direct relationship between colony count and the amount of mutagen. Agar incorporation tests can be used to generate dose–response curves similar to that shown in Fig. 34.7. The simplicity, sensitivity and reproducibility of the Ames test has resulted in its widespread use for screening potential carcinogens in many countries, though it is not an infallible test for carcinogenicity.

SAFETY NOTE Correct handling procedures *must* be followed at all times, as the test substances may be carcinogenic – testers should wear gloves and avoid skin contact or ingestion.

- The standards should be chemically identical to the compound being measured and spread over the expected concentration range being tested.
- The samples should be purified if interfering compounds are present and diluted so the response will be on the 'linear' portion of the assay curve.

Taking account of legal issues when using animal bioassays – in the UK, where bioassays involving 'higher' (vertebrate) animals are controlled by the *Animals Scientific Procedures Act (1986) Amendment Regulations (2012)*, they can only be carried out under the direct supervision of a scientist licensed by the Home Office.

To check for interference, you can standardise the bioassay against another method (preferably physicochemical). Related compounds known to be present in the analyte solution should be shown to have minimal activity in the bioassay. If an interfering compound is present, this may show up if a known amount of standard is added to sample vials – the result will not be the sum of independently determined results for the standard and sample.

Text reference

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Sources for further study

Aschner, M. Sunol, C. and Bal-Price A.K. (2011) *Cell Culture Techniques*. Humana Press, London.

Helgason, C.D. and Miller, C. (2013), *Basic Cell Culture Protocols*, 4th edn. Human Press, London.

STUDY EXERCISES

34.1 Calculate the specific growth rate and doubling time of cells in culture. What are the specific growth rates and doubling times of the following (give all answers to three significant figures)?

- (a) A broth culture of the yeast *Saccharomyces cerevisiae* growing in exponential phase and containing 5.2×10^4 cells at 10 a.m. and 3.4×10^6 cells at 7 p.m.
- (b) A log phase culture of *Escherichia coli* containing 3.0×10^4 CFU mL⁻¹ at the start of the experiment and 6.7×10^7 CFU mL⁻¹ at the end of the experiment, 200 min later.
- (c) An actively growing culture of *Bacillus subtilis* containing 32 bacteria in 25 squares of a haemocytometer chamber at 13.00 h and 250 bacteria in 25 squares of the same haemocytometer chamber at 15.30 h.

34.2 Practise the calculations involved in using a haemocytometer to make a direct microscopic count. Express your answers to three significant figures in all cases.

- (a) The mean cell count of a yeast suspension per small square of an improved Neubauer counting

chamber was 6.42. If the volume of a small square is 2.5×10^{-7} mL, what is the cell count per mL?

- (b) The following counts were obtained for bacteria in 20 individual small squares of a Petroff-Hauser counting chamber: 26, 36, 42, 35, 27, 16, 29, 50, 24, 43, 41, 35, 18, 36, 33, 47, 25, 46, 32, 57. If the volume of each small square is 8×10^{-7} mL, what is the cell count per mL?
- (c) A 10^{-2} dilution of a dense suspension of *E. coli* was examined microscopically using an improved Neubauer counting chamber, giving a total of 78 cells in a total of 25 small squares. If the volume of each small square is 2.5×10^{-7} mL, what is the cell count per mL of the original (undiluted) suspension.
- (d) A dilute suspension of yeast cells was concentrated ten-fold by centrifuging 10 mL and resuspending the pellet in 1 mL. The mean cell count of the concentrated yeast suspension per large square of a Petroff-Hauser counting chamber was 6.8. If the volume of the large square is 2×10^{-5} mL, what is the cell count per mL of the original suspension?

34.3 Practise the calculations involved in making a plate count. Express your answers to three significant figures.

- (a) The mean spread-plate count for 100 μL of a 10^{-5} dilution of a culture of *E. coli* was 54.4 CFU. What is the mean plate count per mL of the original suspension?
- (b) Three replicate plates of nutrient agar were each spread with 200 μL of a 10^{-3} dilution of a bacterial suspension, giving colony counts of 34, 40 and 37 after 24 h incubation. What is the mean plate count per mL of the original suspension?
- (c) A twenty-fold dilution of a yeast suspension was used to prepare four replicate pour plates, each containing 500 μL of this dilution and giving counts of 211, 186, 194 and 202 after incubation. What is the mean plate count per mL of the original suspension?
- (d) A sample of 50 g of raw seafood was homogenised and diluted to 2% w/v in sterile saline solution. Three replicate pour plates were prepared using 1 mL of the diluted sample, giving counts for *E. coli* of 35, 41 and 32. What is the average count of *E. coli* per 100 g of raw seafood?
- (e) Duplicate samples of 250 mL of river water were filtered through separate sterile membranes of pore size 0.2 μm . The membranes were then transferred to the surface of an agar-based medium and incubated for 48 h at 37 °C, giving colony counts of 45 and 32 respectively. What is the mean count per 100 mL of river water?
- (f) A sample of bottled drinking water was processed by serial decimal dilution and 500 μL samples of each dilution were pour-plated and incubated at 22 °C for 72 h. The colony counts obtained for the three replicate plates of the 10^{-1} dilution were 28, 32 and 39. Does this water meet the EU regulations for mineral water, which specify a maximum average plate count at 22 °C of 100 CFU mL $^{-1}$?

34.4 Use bioassay data to estimate the amount of analyte in unknown samples. Bioassay data obtained by exposing water fleas (*Daphnia* sp.) to a purified algal toxin are shown in the table below.

- (a) Graph the data and use the graph to estimate the toxin contents of two samples obtained from two lochs which gave (i) a heart rate of 130 beats per minute and (ii) a heart rate of 25 beats per minute.
- (b) Which one of the two estimates would be the least reliable and why?
- (c) How could you improve the reliability of this estimate if you had more of the sample?

- (d) What unmeasured factors might limit the usefulness of this assay?

Responses of *Daphnia* to toxin.

Toxin concentration (mmol L $^{-1}$)	Mean heart rate of <i>Daphnia</i> sp. (beats per minute)
1.00×10^{-3}	20
3.16×10^{-4}	23
1.00×10^{-4}	26
3.16×10^{-5}	50
1.00×10^{-5}	180
3.16×10^{-6}	192
1.00×10^{-6}	195

34.5 Use agar diffusion bioassay data to quantify a growth inhibitor in a sample. Agar diffusion bioassay is a simple means of estimating the amount of an inhibitory substance in a test sample, by adding the substance, in aqueous solution, to wells cut in a Petri plate of agar medium seeded with a microbe sensitive to the substance and then incubating the plate at an appropriate growth temperature. Diffusion of the inhibitory substance gives a zone of growth inhibition proportional to \log_{10} of the concentration of substance. In the following example and table (below), the amount of the antibiotic nisin in a test sample can be determined by comparing the degree of growth inhibition of *Bacillus subtilis* for the test solution and a series of standards containing known concentrations of nisin. Prepare a calibration curve and determine the nisin concentration of solution 5 (unknown). Express your answer in $\mu\text{g mL}^{-1}$, to one decimal place.

Growth inhibition of *B. subtilis* in solutions containing different amounts of nisin.

Solution	Nisin content (ng mL $^{-1}$)	Zone of growth inhibition (mm)
1	40	26.5
2	20	23
3	10	19.5
4	5	17
5	unknown (test solution)	22.5

Answers to these study exercises are available at go.pearson.com/uk/he/resources

35 Collecting and isolating microbes

Using alternatives to traditional culture-based methods – the presence of microbes in the environment can be studied by molecular methods, including the amplification of specific nucleic acid sequences by PCR (Chapter 68), which reveal their 'molecular signature'.

Avoiding contamination during sampling – always remember that you are the most important source of contamination of field samples: components of the oral or skin microbiota are the most likely contaminants.

Subsampling – to minimise the effects of changes in temperature, aeration and water status during transportation, a primary sample may be returned to the laboratory, where the working sample (subsample) is then taken (e.g. from the centre of a large block of soil).

SAFETY NOTE When working with newly isolated microbes, you should always treat them as potentially harmful until they have been identified (Chapter 36).

Microbes can be studied by taking samples from the environment, including water, soil, air and the microbiota of the skin and other sites. Standard methods for analysing bacterial, fungal and virus samples include:

- **direct examination of individual cells** of a particular microbe, for example, using fluorescence microscopy (p. 171)
- **isolation/purification of a particular species or related individuals** of a taxonomic group, for example, the faecal indicator bacterium *Escherichia coli* in sea water
- **the study of microbial processes**, rather than individual microbes, either *in situ*, or in the laboratory.

Processing samples

Sampling techniques include the use of swabs, sticky tape, strips and agar contact methods for sampling surfaces, bottles for aquatic habitats, plastic bags and corers for soils and sediments. A wide range of complex apparatus is available for accurately sampling water or soil at particular depths.

KEY POINT An important feature of all microbiological sampling protocols is that the sampling apparatus must be sterile; strict aseptic technique must be used throughout the sampling process (see Chapter 33).

The sampling method must minimise the chance of contamination with microbes from other sources, especially the exterior of the sampling apparatus and the operator. For example, if you are sampling an aquatic habitat, stand downstream of the sampling site. A portable Bunsen burner or spirit lamp can be used to assist sterile technique during field sampling, for example, while flaming a loop (p. 236). Alternatively, use disposable plastic loops.

Process the sample as quickly as possible, to limit any changes in microbiological status. As a general guideline, many procedures require samples to be analysed within six hours of collection. Changes in aeration, pH and water content may occur after collection. Some microbes are more susceptible to such effects. For example, anaerobic bacteria may not survive if the sample is exposed to air. Sunlight can also damage bacteria; samples should be shielded from direct sunlight during collection and transport to the laboratory.

Soil and water samples are often kept cool (at 0–5 °C) during transport to the laboratory. In contrast, some microbes adapted to grow in association with warm-blooded animals may be damaged by low temperatures. An alternative approach is to keep the sample near the ambient sampling temperature using an insulated vessel (for example, a Thermos flask).

Isolating and purifying techniques

Several different approaches may be used to obtain microbes in pure culture. The choice of method will depend upon the microbe to be isolated: some organisms are relatively easy to isolate, while others require more involved procedures.

Separation methods

Most microbial isolation procedures involve some form of separation to obtain individual microbial cells. The most common approach is to use an agar-based medium for primary isolation, with streak dilution, spread plating or pour plating to produce single colonies, each derived from a single type of microbe (pp. 273–4). It is often necessary to dilute samples before isolation, so that a small number of individual microbial cells is transferred to the growth medium. Strict serial dilution (pp. 147–8) of a known amount of sample is needed for quantitative work.

Obtaining a pure culture – if a single colony from a primary isolation medium is used to prepare a streak dilution plate and all the colonies on the second plate appear identical, then a pure culture has been established. Otherwise, you cannot assume that your culture is pure and you should repeat the subculture until you have a pure culture.

Using a sonicator – minimise heat damage with short treatment ‘bursts’ (typically up to 1 min), cooling the sample between bursts, e.g. using ice.

Definitions

Mesophile – a microbe with an optimum growth temperature of 20–45 °C (lit. ‘middle-loving’).

Psychrophile – a microbe with an optimum temperature for growth of <20°C (lit. ‘cold-loving’).

Psychrotroph – a microbe with an optimum temperature for growth of ≥20°C, but capable of growing at lower temperature, typically 0–5 °C (lit. ‘cold-feeding’).

Thermophile – a microbe with an optimum growth temperature of >45 °C (lit. ‘heat-loving’).

KEY POINT If your aim is to isolate a particular microbe, perhaps for further investigation, you will need to subculture individual colonies from the primary isolation plate to establish a pure culture, also known as an axenic culture.

Pure cultures of most microbes can be maintained indefinitely, using sterile technique and microbial culture methods (Chapters 33 and 34).

Other separation techniques include:

- **Dilution to extinction.** This involves diluting the sample to such an extent that only one or two microbes are present per millilitre: small volumes of this dilution are then transferred to a liquid growth medium (broth). After incubation, most of the tubes will show no growth, but some tubes will show growth, having been inoculated with a single viable microbe at the outset. This should give a pure culture, though it is wasteful of resources.
- **Sonication/homogenisation.** This is useful for separating individual microbial cells from each other and from inert particles, prior to isolation. However, some decrease in viability is likely.
- **Filtration.** This can be useful where the number of microbes is low. Samples can be passed through a sterile cellulose ester filter (pore size 0.2 µm), which is then incubated on the surface of an appropriate solidified medium. Sieving and filtration techniques are often used in soil microbiology to subdivide a sample on the basis of particle size.
- **Micromanipulation.** It may be possible to separate a microbe from contaminants using a micropipette and dissecting microscope (pp. 143–4 and pp. 181–2). The microbe can then be transferred to an appropriate growth medium, to give a pure culture. However, this is rarely an easy task for the novice.
- **Motility.** Phototactic microbes (including photosynthetic flagellates and motile cyanobacteria) will move towards a light source; heterotrophic flagellate bacteria will move through a filter of appropriate pore size into a nutrient solution, or away from unfavourable conditions (positive or negative chemotaxis).

Selective and enrichment methods

Selective and enrichment techniques can be considered together, since they both enhance the growth of a particular microbe when compared with its competitors and they are often combined in specific media (see Box 38.1).

The difference between selective and enrichment techniques is that the former use growth conditions unfavourable for competitors while the latter provide improved growth conditions for the chosen microbes. Laboratory incubation under selective or enrichment conditions should allow the particular microbes to be isolated in pure culture.

KEY POINT Selective methods are based on the use of physicochemical conditions that will permit the growth of a particular group of microbes while inhibiting others. Enrichment techniques encourage the growth of certain bacteria, usually by providing additional nutrients in the growth medium.

Methods based on specific physical conditions include:

- **Temperature.** Psychrophilic and psychrotrophic microbes can be isolated by incubating the growth medium at 4 °C, while thermophilic microbes require temperatures above 45 °C for isolation. Short-term heat treatment of samples can be used to select for endospore-forming bacteria, for example, 70–80 °C for 5–15 min, prior to isolation.
- **Atmosphere.** Many eukaryotic microbes are obligate aerobes, requiring an adequate supply of oxygen to grow. Bacteria vary in their responses to oxygen: obligate anaerobes are the most demanding, growing only under anaerobic conditions (for example, in an anaerobic cabinet or jar). Oxygen requirements can be determined using the agar shake tube method as part of the isolation procedure (Fig. 35.1). Some pathogenic bacteria grow best in an atmosphere with a reduced oxygen status and increased CO₂ concentration: such carboxyphilic bacteria (capnophiles) are grown in an incubator where the gas composition can be adjusted.
- **Centrifugation.** This can be used to separate buoyant microbes from their non-buoyant counterparts – on centrifugation, such organisms will collect at the surface while the remaining microbes will sediment. Alternatively, density gradient methods may be used (pp. 320–1). Centrifugation can be combined with repeated washing, to separate microbes from contaminants.
- **Ultraviolet irradiation.** Some microbes are tolerant of UV treatment and can be selected by exposing samples to UV light. However, the survivors may show a greater rate of mutation.
- **Illumination.** Samples may be enriched for cyanobacteria and microalgae by incubation under a suitable light regime. For dilute samples, where the number of photosynthetic microbes is too low to give the sample any visible green coloration, there is a risk of photoinhibition and loss of viability if the irradiance is too high. Such samples need shading during initial growth.

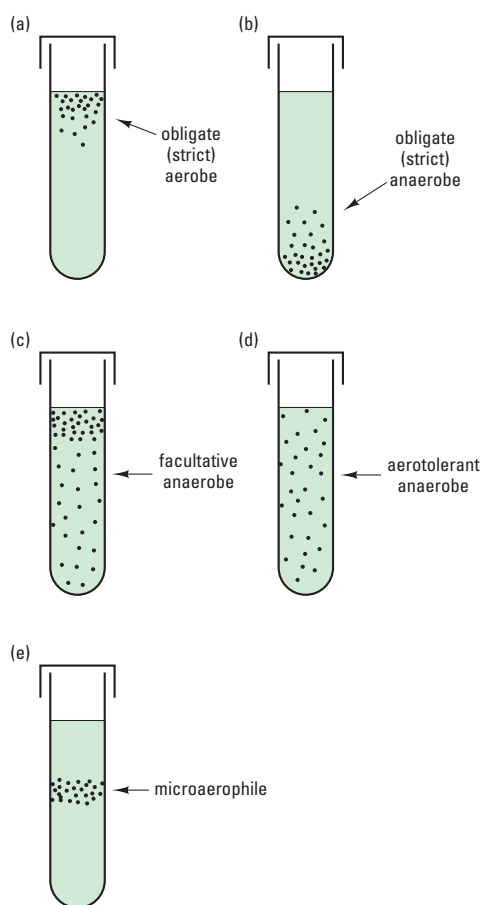


Fig. 35.1 Agar shake tubes. Bacteria are suspended in molten agar at 45–50 °C and allowed to cool. The growth pattern after incubation reflects the atmospheric (oxygen) requirements of the bacterium.

Chemical methods form the mainstay of bacteriological isolation techniques and various media have been developed for the isolation of specific groups of bacteria. The chemicals involved can be subdivided into the following groups:

- **Selectively toxic substances:** for example, salt-tolerant, Gram-positive cocci can be grown in a medium containing 7.5% w/v NaCl, which prevents the growth of most common heterotrophic bacteria.

Definitions

Aerotolerant anaerobe – a microbe that grows by fermentation, but which is insensitive to air/oxygen (in contrast to strict anaerobes, which are typically killed by exposure to air/oxygen).

Capnophile – a microbe that thrives in the presence of high levels of atmospheric carbon dioxide.

Facultative anaerobe – a microbe that grows by aerobic respiration when oxygen is present, switching to fermentation under anaerobic conditions.

Several media include dyes as selective agents, particularly against Gram-positive bacteria.

- **Antibiotics:** for example, the use of antibacterial agents (for example, penicillin, streptomycin, chloramphenicol) in media designed to isolate fungi, or the use of antifungal agents (for example, cycloheximide, nystatin) in bacterial media. Some antibacterial agents show a narrow spectrum of toxicity and these can be incorporated into selective isolation media for resistant bacteria, for example, metronidazole for anaerobic bacteria.
- **Nutrients that encourage the growth of certain microbes:** including the addition of a particular carbon source, or specific inorganic nutrients.
- **Substances that affect the pH of the medium:** for example, the use of alkaline peptone water at pH 8.6 for the isolation of *Vibrio* spp.

KEY POINT Note that subcultures from a primary isolation medium must be grown in a non-selective medium, to confirm the purity of the isolate.

Table 35.1 Selective agents in bacteriological media

Substance	Selective for
Azide salts	<i>Enterococcus</i> spp.
Bile salts	Intestinal bacteria
Brilliant green	Gram-negative bacteria
Gentian violet	Gram-negative bacteria
Lauryl sulphate	Gram-negative bacteria
Methyl violet	<i>Vibrio</i> spp.
Malachite green	<i>Mycobacterium</i>
Polymyxin	<i>Bacillus</i> spp.
Sodium selenite	<i>Salmonella</i> spp.
Sodium chloride	Halotolerant bacteria <i>Staphylococcus aureus</i>
Sodium tetrathionate	<i>Salmonella</i> spp.
Trypan blue	<i>Streptococcus</i> spp.
Tergitol/surfactant	Intestinal bacteria

Many of the selective and enrichment media used in bacteriology allow you to distinguish between different types of bacteria: such media are termed differential media or diagnostic media and they are often used in the preliminary stages of an identification procedure. Box 38.1 gives details of the constituents of MacConkey medium, a selective, differential medium used in clinical microbiology (for example, for the isolation of certain faecal bacteria), while Table 35.1 gives details of selective agents used. Further details on methods can be found in Collins *et al.* (2004). Note that isolation procedures for a particular microbe often combine several of the techniques described above. For instance, a protocol for isolating food-poisoning bacteria from a foodstuff might involve:

1. **homogenisation** of a known amount of sample in a suitable diluent
2. **serial decimal dilution**
3. **separation procedures** using spread or pour plates to quantify the number of bacteria of a particular type present in the foodstuff and provide a viable count
4. **selective/enrichment procedures**, for example, specific media/temperatures/ atmospheric conditions, depending on the bacteria to be isolated
5. **confirmation of identity:** any organism growing on a primary isolation medium would require subculture and further tests, to confirm the preliminary identification (Chapter 36).

Text reference

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STUDY EXERCISES

35.1 Plan a collection/sampling strategy for a target group of microbes. What approaches might you take in the following instances?

- (a) Collecting representatives of the normal skin microflora.
- (b) Sampling psychrotrophic anaerobes from the subsurface mud of an estuary.
- (c) Collecting a sample of sea water to enumerate faecal indicator bacteria.
- (d) Mapping the microflora on the surface of a leaf.

35.2 Decide on an appropriate isolation procedure for a particular microbe. How might you isolate the following microbes from a sample?

- (a) Photosynthetic flagellate algae in a sample of pond water.
- (b) Bacteria growing as a biofilm on the surface of sand particles.

- (c) Faecal streptococci (enterococci) present at low density (<1 per mL) in a sample of river water.
- (d) A distinctively shaped bacterium present at very low numbers in a sample containing a high number of other microbes of different shape but with similar nutritional requirements.

35.3 Investigate the selective basis of microbiological media. Using textbooks on bacteriological methods or the Web, research how each of the following media operates, in terms of their selective and diagnostic (differential) features:

- (a) Mannitol salt agar for *Staphylococcus aureus*.
- (b) Membrane lauryl sulphate broth for coliforms and *E. coli*.
- (c) Slanetz and Bartley medium for faecal streptococci (enterococci).
- (d) Mannitol pyruvate egg yolk polymyxin (MPYP) medium for *Bacillus cereus*.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

36 Identifying microbes

Microbial identification typically involves traditional morphological and culture-based methods in tandem with more recent molecular approaches. You are most likely to develop your skills using bacterial examples to illustrate the general principles. While relevant techniques can be applied to many other cellular microbes, including fungi and protists (Chapter 39), virus identification requires nucleic acid sequencing, electron microscopy or immunological techniques (Chapter 40).

KEY POINT Identification of bacteria is often based on a *combination* of a number of different aspects, including growth characteristics, microscopic features, physiological and biochemical characterisation, and, where necessary, immunological tests and nucleic acid analysis.

Observing growth forms

Once a microbe has been isolated (Chapter 35) and cultured in the laboratory (Chapter 34), the appearance of individual colonies on the surface of a growth medium solidified with agar often provides useful information. Bacteria typically produce smooth, glistening colonies up to 1 cm in diameter. Actinomycete colonies are often <1 cm, with a shrivelled, powdery, matt surface. Filamentous fungi (Chapter 39) usually grow as large, spreading mycelia composed of microscopic branching filaments (hyphae) with a matt appearance, traditionally identified by the microscopic appearance of their reproductive structures. Yeasts produce small, glistening colonies; further identification usually involves microscopy, combined with physiological and biochemical tests similar to those used for bacteria.



Fig. 36.1 Colony elevations (cross-sectional profile).

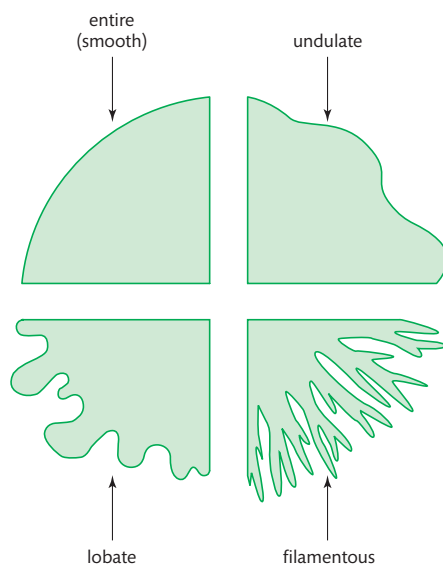


Fig. 36.2 Colony margins (surface view).

Colony characteristics

When measuring colony size, choose a typical colony, well spaced from any others, as size is affected by competition for nutrients. Record the following characteristics:

- **Size:** some bacteria produce pin-point (punctiform) colonies, ≤ 1 mm in diameter, while motile bacteria may spread over the entire plate.
- **Form:** colonies may be circular, irregular, spindle-shaped (lenticular) or filamentous.
- **Elevation (height):** colonies may be flat, raised, convex, etc. (see Fig. 36.1).
- **Margin:** the edge of a colony may be entire (smooth) or more distinctive, for example, undulate, lobate or filamentous (Fig. 36.2).
- **Consistency:** colonies may be viscous (mucoid), butyrous (of similar consistency to butter) or friable (dry and granular).
- **Colour:** some bacteria produce characteristic pigments. A few pigments are fluorescent under UV light.
- **Optical properties:** colonies may be translucent or opaque.
- **Haemolytic reactions on blood agar:** many pathogenic bacteria produce characteristic zones of haemolysis due to breakdown of haemoglobin.

SAFETY NOTE Take care not to smell mould cultures, because of the risk of inhaling large numbers of spores, which may cause an allergic reaction.

Using the hanging drop technique – place a drop of bacterial suspension on a coverslip and invert over a cavity slide so that the drop does not make contact with the slide: motile aerobes are best observed at the edge of the drop, where oxygen is most abundant.

Assessing motility – if you have not seen bacterial motility before, it is worth comparing your unknown bacterium with a positive and a negative control.

Using cell shape in microbial identification – many bacteria are pleomorphic, varying in size and shape according to growth conditions and age of culture: thus, other characteristics are required for identification.

Assessing the Gram status of an unknown bacterium – if a pure culture shows both Gram-positive and Gram-negative cells, it is either (i) as a Gram-positive organism that is demonstrating Gram-variability or (ii) due to poor technique.

Alpha haemolysis is a partial breakdown, producing a green zone around the colony, while beta haemolysis is the complete destruction of haemoglobin, producing a clear zone.

- **Odour:** while some actinomycetes produce ‘earthy’ odours, due to the presence of geosmin, and certain bacteria produce fruity or ‘off’ smells, odour is not a reliable characteristic and its use is not recommended on safety grounds.

Examining specimens microscopically

Observe bacteria using an oil immersion objective at a total magnification of $\times 1000$ (p. 178).

Assessing motility

Prepare a wet mount by placing a small drop of bacterial suspension on a clean slide, adding a coverslip and examining the film without delay. For aerobes, areas near air bubbles or by the edge of the coverslip give best results, whereas anaerobes show greatest motility in the centre of the preparation, with rapid loss of motility due to oxygen toxicity. Determine motility using cultures in exponential growth in a liquid medium (pp. 242–3). It is best to work with cultures grown at 20 or 25 °C, since those grown at 37 °C may not be actively motile on cooling to room temperature. It is essential to distinguish between the following:

- **true swimming motility**, due to the presence of flagella: bacteria dart around the field of view, changing direction in zigzag, tumbling movements
- **Brownian motion:** non-motile bacteria show a vibratory, random motion, due to bombardment by molecules in solution
- **passive motion**, due to currents within the suspension: all cells will be swept in the same direction at a similar rate of movement
- **gliding motility:** a slower, intermittent movement, parallel to the longitudinal axis of the cell, requiring contact with a solid surface.

Cell shape

Describe bacteria using the following morphological terms:

- **cocci** (singular, coccus): spherical, or almost spherical, cells, sometimes growing in pairs (diplococci), chains or clumps
- **rods:** straight, cylindrical cells of variable length, with flattened, tapered or rounded ends; sometimes termed bacilli. Short rods are sometimes called cocco-bacilli
- **curved rods:** the curvature and length varies from short comma-shaped rods (vibrios), to longer spiral/helical shapes (for example, spirochaetes)
- **branched filaments:** characteristic of actinomycetes.

Gram staining

This is the most important differential staining technique in bacteriology (Box 36.1 gives details). It enables us to divide bacteria into two distinct groups, Gram-positive and Gram-negative, according to a particular staining procedure (the technique is given a capital letter after its originator, H.C. Gram). The basis of the technique is the different structure of the

Box 36.1 How to prepare a heat-fixed, Gram-stained smear of a bacterial culture**Preparing a heat-fixed smear**

Use the following procedure to achieve a thin smear of bacteria on a microscope slide, for staining.

1. **Take a clean microscope slide and pass through a Bunsen flame twice, to degrease.** Allow to cool.
2. **Using a sterile inoculating loop (p. 237), place a single drop of water in the centre of the slide and then mix with a small amount of sample from a single bacterial colony,** until the suspension is slightly turbid. Smear over the central area of the slide, to form a thin film. For liquid cultures, use a single drop of culture fluid, spread in a similar manner.
3. **Allow to air-dry at room temperature,** or high above a Bunsen flame: air-drying must be carried out gently, or the cells will shrink and become distorted.
4. **Fix the air-dried film by passage through a Bunsen flame.** Using a slide holder or forceps, pass the slide, film side up, rapidly through the hottest part of the flame (just above the blue cone). Note that you must not overheat the slide or you will ruin the preparation.
5. **Allow to cool:** the smear is now ready for staining.

Gram-staining procedure

This version is based on Hucker's modification of the method.

SAFETY NOTE Some of the staining solutions used are flammable, especially the acetone decolourising solvent: make sure that all Bunsens are turned off during staining. Disposable gloves can be used to prevent skin contact with toxic dyes.

Carry out Gram staining with your slide suspended over a sink, on a staining rack. Take care not to invert the slide during processing, or before microscopy.

1. **Flood a heat-fixed smear with 2% w/v crystal violet in 20% v/v ethanol:water** and leave for 1 min.
2. **Pour off the crystal violet and rinse briefly with tap water. Flood with Gram's iodine** (2 g KI and 1 g I₂ in 300 mL water) for 1 min.
3. **Rinse gently with tap water.** Leave the tap running.
4. **Tilt the slide and decolourise with acetone** for a couple of seconds: add acetone dropwise to the slide until no colour appears in the effluent. Timing is critical, since acetone is a powerful decolourising solvent and must not be left in contact with the slide for too long.
5. **Immediately immerse the slide in a gentle stream of tap water,** to remove the acetone.
6. **Pour off the water and counterstain for around 30 s using 2.5% w/v safranin** in 95% v/v ethanol:water.
7. **Pour off the counterstain, rinse briefly with tap water, then dry the smear** by blotting gently with absorbent paper: all traces of water must be removed before the stained smear is examined microscopically.
8. **Place a small drop of immersion oil on the stained smear: examine directly** (without a coverslip) using an oil-immersion objective (p. 178).

Gram-positive bacteria retain the crystal violet (primary stain) and appear purple while Gram-negative bacteria are decolourised by acetone and counterstained by the safranin, appearing pink or red when viewed microscopically.

Other decolourising solvents are sometimes used, with adjustment to the timing depending upon the strength of the solvents (e.g. ethanol-based solvents require around 30 s to decolourise a smear).

Staining bacterial spores – these are relatively impermeable to dyes, and require heat treatment and specialised stains.

cell walls of Gram-positive and Gram-negative bacteria. Heat fixation of air-dried bacteria causes some shrinkage, but cells retain their shape: to measure cell dimensions use a chemical fixative.

Gram staining should be carried out using light smears of young, active cultures, since older cultures may give variable results. In particular, older cultures of Gram-positive bacteria may stain Gram-negative, due to autolytic changes in their cell walls. Developing spores are often visible as unstained areas within older vegetative cells of *Bacillus* and *Clostridium*. Other stains are required to demonstrate sub-cellular structures such as, capsules or flagella (p. 180).

Performing the oxidase test – never use a nichrome wire loop, as this will react with the oxidase reagent, giving a false positive result.

SAFETY NOTE The catalase and oxidase reagents are irritants and could be harmful if swallowed. Avoid skin contact and ingestion.

Avoiding false negatives – ensure you use sufficient material during oxidase and catalase testing; otherwise you may obtain a false negative result: a clearly visible ‘clump’ of bacteria should be used.

Diagnosing via carbohydrate utilisation tests and isolation media – many agar-based media incorporate one or more specific carbohydrates and pH indicator dyes, providing indicative biochemical information as part of the isolation procedure.

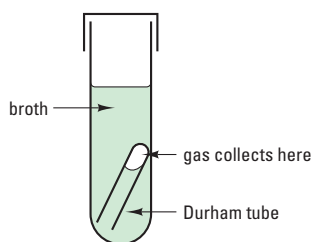


Fig. 36.3 Durham tube in carbohydrate utilisation broth. Air within the Durham tube is replaced by broth during the autoclaving procedure. Broth is then displaced by CO₂ gas produced during carbohydrate breakdown.

Using basic laboratory tests

You are most likely to use two simple biochemical tests in bacterial identification:

- **Oxidase:** this identifies cytochrome *c* oxidase in obligate aerobes. Soak a small piece of filter paper in a fresh solution of 1% (w/v) *N-N'-N''-N'''*-tetramethyl-*p*-phenylenediamine dihydrochloride (‘oxidase reagent’) on a clean microscope slide. Rub a small amount from the surface of a young, active colony onto the filter paper using a glass rod, a *plastic* loop or a wooden applicator/toothpick: a purple-blue colour within 10 s is a positive result.
- **Catalase:** This identifies an enzyme found in both obligate aerobes and in most facultative anaerobes, catalysing the breakdown of hydrogen peroxide into water and oxygen ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$). Transfer a small sample of your unknown bacterium on to a coverslip using a disposable plastic loop or glass rod. Invert on to a drop of hydrogen peroxide (catalase reagent) on a slide: the appearance of bubbles within 30 s is a positive reaction. This method minimises the dangers from aerosols formed when oxygen bubbles burst.

These two tests allow us to subdivide bacteria on the basis of their oxygen requirements, since, in broad terms:

- **obligate aerobes will be oxidase-positive and catalase-positive**
- **facultative anaerobes will be oxidase-negative and catalase-positive**
- **microaerophilic bacteria, aerotolerant anaerobes and strict (obligate) anaerobes will be oxidase-negative and catalase-negative** – the latter group will grow only under anaerobic conditions (p. 254).

Once you have reached this stage (colony characteristics, motility, shape, Gram reaction, oxidase and catalase status), it may be possible to make a tentative identification, at least for certain Gram-positive bacteria, at the generic level. To identify Gram-negative bacteria, particularly the oxidase-negative, catalase-positive rods, further tests are required.

Using identification tables: further laboratory tests

Bacteria are asexual organisms and strains of the same species may give different results for individual biochemical/physiological tests. This variation is allowed for in identification tables (multi-access keys, pp. 261), based on the results of a large number of tests. Identification tables are often used for particular subgroups of bacteria, after basic laboratory tests have been performed: an example is shown in Table 36.1.

A large number of specific biochemical and physiological tests are used for different subgroups. These tests include:

- **Carbohydrate utilisation tests.** Some bacteria can use a particular carbohydrate as a carbon and energy source. Acidic end products can be identified using a pH indicator dye (p. 162) while CO₂ is detected in liquid culture using an inverted small test tube (Durham tube, Fig. 36.3). Aerobic breakdown (via respiration) is termed oxidation, and anaerobic breakdown is known as fermentation. Identification tables usually incorporate tests for several different carbohydrates, for example, Table 36.1.

Table 36.1 Identification table for selected Gram-negative rods

Bacterium	Biochemical test								
	1	2	3	4	5	6	7	8	9
<i>Escherichia coli</i>	v	+	–	+	–	v	v	+	–
<i>Proteus mirabilis</i>	–	–	v	–	+	–	+	–	+
<i>Morganella morganii</i>	–	–	–	–	+	–	+	+	–
<i>Vibrio parahaemolyticus</i>	–	+	v	–	–	+	+	+	–
<i>Salmonella spp.</i>	–	+	v	–	–	+	+	–	+

Key to biochemical tests and symbols:

- | | |
|---|--|
| 1. sucrose utilisation | 7. ornithine decarboxylase activity |
| 2. mannitol utilisation | 8. indole production |
| 3. citrate utilisation | 9. H ₂ S production |
| 4. β-galactosidase activity | +, >90% of strains tested positive |
| 5. urease activity | –, <10% of strains tested positive |
| 6. lysine decarboxylase activity | v, 10–90% of strains tested positive |

Understanding biochemical identification

– given the variability of different isolates of a single species, a large number of tests must be used to provide a high degree of probability of correctly identifying the isolate. This is often carried out in specialised labs using computer-aided analysis.

- **Enzyme tests.** Most of these incorporate a substance that changes colour if the enzyme is present, typically a chromogenic substrate.
- **Tests for specific end products of metabolism,** for example, the production of indole due to the metabolic breakdown of the amino acid tryptophan, or H₂S from sulfur-containing amino acids.

Using identification kits

Some biochemical tests are now supplied in kit form; for example, the API 20E system incorporates 20 tests within a sterile plastic strip (Fig. 36.4). After inoculation and overnight incubation, the results of the tests are converted into a seven-digit code, for comparison with known bacteria using dedicated software (for example, the Analytical Profile Index). While kit identification systems save time and labour, they are more expensive and less flexible than conventional biochemical tests.

Using antibiotic resistance to identify bacteria

– patterns of resistance to antibacterial agents in lab tests can be used to generate an 'antibiogram', giving information that can aid identification, as well as providing data of clinical significance, e.g. for therapy of bacterial infections.

Using immunological tests

Tests used in diagnostic microbiology include:

- **Agglutination tests:** based on the reaction between specific antibodies and a particular bacterium (p. 393). These tests are particularly useful for subdividing biochemically similar bacteria.
- **Fluorescent antibody tests:** the reaction between a labelled antibody and a particular bacterium can be visualised using UV microscopy. The

Fig. 36.4 Example of a bacterial identification kit (API 20E).

Property of bioMérieux S.A./Andrea Bannuscher.



direct fluorescent antibody test uses fluorescein isothiocyanate as the label.

- **Enzyme-linked immunoassay tests** using antibodies labelled with a particular enzyme, for example, the ELISA tests described on pp. 396–7.

While immunological tests can give specific and accurate confirmation of the identity of an unknown bacterium, they are often too expensive and time-consuming for routine identification, especially when large numbers of tests are required, as in medical diagnostics.

Using nucleic acid tests

Methods based on molecular biology are becoming more widely used, due to a combination of speed, sensitivity and specificity. While there are many variations, the process usually involves two main aspects:

- **Amplification of a specific nucleic acid sequence of nucleic acid (either DNA or RNA) from the target microbe** using the polymerase chain reaction (PCR, pp. 396–7). This is particularly useful for microbes in environmental samples and those with unknown culture requirements.
- **Detection of the target nucleic acid.** This can be achieved by hybridisation of the target nucleic acid sequence in single-stranded format with a single-stranded oligonucleotide ‘probe’ (p. 517) of complementary sequence that also includes a ‘reporter molecule’ – for example, a fluorescent dye. In the nucleic acid ‘dot blot’ process, a nitrocellulose filter is used as support medium for the hybridisation step, removing the need for electrophoresis and Southern blotting (p. 517). Irrespective of method, positive and negative control should be tested, to check sensitivity and specificity. Consult Chapter 67 for further details of methods.

Understanding sensitivity and specificity in testing – the sensitivity of a test is its ability to correctly identify a positive result, whereas specificity is its ability to correctly identify a negative result.

Practical applications of bacterial typing – *E. coli* O157:H7 is a serotype of this bacterium that is capable of causing severe human disease: it can be identified on the basis of an agglutination reaction with an appropriate antiserum. Typing is explained further on p. 267.

Typing methods (subspecies identification)

The identification of bacteria at subspecies level is known as typing: this is usually done in a specialist laboratory, for example, as part of an epidemiological study to establish the source of an infection. The methods used include:

- **antigen typing** (serotyping): determined using immunological tests
- **phage typing**: based on susceptibility to certain bacterial viruses (phages)
- **biotyping**: based on biochemical differences between different strains
- **bacteriocin typing**: using inhibitory proteins released by some strains of bacteria.

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STUDY EXERCISES

36.1 Describe the colonial characteristics of selected microbes. Either research the features of the following microbes (e.g. via the Web), or look at well-isolated individual colonies in the laboratory, following overnight growth on a suitable medium:

- (a) *Escherichia coli*
- (b) *Pseudomonas aerogenes*
- (c) *Staphylococcus aureus*
- (d) *Streptococcus pneumoniae*
- (e) *Bacillus subtilis*.

36.2 Find out how some of the tests used in microbial identification work. Research the operating principles that underpin the following tests, using microbiological textbooks or the Web, and prepare brief notes explaining how each of the following tests works:

- (a) indole test
- (b) β -galactosidase test

- (c) urease test
- (d) lysine decarboxylase test
- (e) H_2S production.

36.3 Identify the following oxidase-negative, catalase-positive, Gram-negative rod-shaped bacteria, using Table 36.1.

- (a) positive for citrate, urease, ornithine decarboxylase and H_2S
- (b) positive for sucrose, mannitol, β -galactosidase, lysine decarboxylase and indole
- (c) positive for mannitol, citrate, lysine decarboxylase, ornithine decarboxylase and H_2S
- (d) positive for urease, ornithine decarboxylase and indole
- (e) positive for mannitol, β -galactosidase and indole.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

37 Naming microbes and other organisms

Definitions

Classification (taxonomy) – the study of the theory and methods of organisation of taxa and, therefore, a part of systematics.

Identification – the placing of organisms into taxa (see Chapter 36).

Nomenclature – the allocation of names to taxa.

Systematics – the study of the diversity of living organisms and of the evolutionary relationships between them.

Taxon (plural taxa) – an assemblage of organisms sharing some basic features.

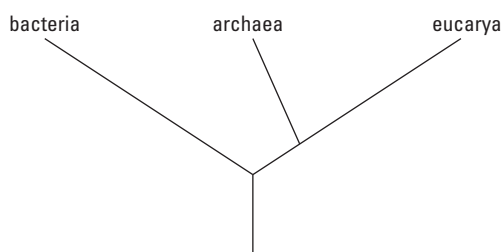


Fig. 37.1 Simplified diagram of the three major domains of the Tree of Life (based on the rRNA sequencing work of Carl Woese).

The use of scientific names is fundamental to all aspects of biological science since it aims to provide a system of identification that is precise, fixed and of universal application. Without such a system, comparative studies would be impossible.

There are two possible bases for such classification:

1. **Phenetic taxonomy**, which involves grouping on the basis of phenotypic similarity, frequently using complex statistical techniques to obtain objective measures of similarity. The characters used have been largely morphological and anatomical, but biochemical, cytological and other characters are increasingly used, especially for microbes (p. 258) where structural characters are few.
2. **Phylogenetic (= phyletic) taxonomy**, which involves grouping on the basis of presumed evolutionary, and therefore genetic, relationships.

These two systems are often broadly similar in outcome, since closely related organisms are usually fairly similar to each other and because judgements of evolutionary relationships are usually themselves based upon similarities. The situation is made more complex by phenomena such as convergent and divergent evolution; phyletic classifications are also liable to subjective bias.

New classifications have been proposed on the basis of studies of biomolecules, for example, rRNA sequences, that are robust and objective (Chapter 67). One such arrangement divides organisms into three major domains: Bacteria, Archaea and Eucarya (Fig. 37.1). These investigations continue to inform classification, where some morphologically similar organisms have been found to be different at the genetic level.

Understanding the hierarchical system

While the details are contested by different authorities, some schemes recognise six kingdoms of cellular organisms:

- **Archaea** (formerly Archaeobacteria). Prokaryotic (lacking a membrane-bound nucleus) and unicellular. Distinguished from bacteria on the basis of 16S rRNA sequencing and other unique attributes (for example, lack of peptidoglycan in their cell walls).
- **Bacteria** (formerly Monera). Prokaryotic and unicellular. Typically contain peptidoglycan in their cell walls. Includes photosynthetic cyanobacteria and heterotrophic forms (Chapter 38).
- **Protists** (Protista). Eukaryotic (with a membrane-bound nucleus) and mainly unicellular. Includes algae, protozoa and slime moulds. A diverse and heterogeneous (polyphyletic) group (pp. 277–8).
- **Plants** (Plantae). Eukaryotic and mainly multicellular. Photoautotrophs – typically non-motile with cellulose cell walls.
- **Fungi**. Eukaryotic and either unicellular or syncytial (pp. 276–7). Heterotrophs – typically walled, with absorptive nutrition (Chapter 39).
- **Animals** (Animalia). Eukaryotic and mainly multicellular. Heterotrophs – typically capable of movement and lacking cell walls.

Viruses are a special case, since they are non-cellular entities that are obligate intracellular parasites (Chapter 40). Some authorities place them in a seventh kingdom, while others exclude them from such classification schemes.

Definition

Saprotroph – a heterotrophic organism that feeds on dead organic matter. (Other nutritional terms are defined on p. 241.)

Using DNA to define a species – in microbiology, members of a bacterial species can be characterised by having a DNA similarity of $\leq 70\%$ based on nucleic acid hybridisation studies (pp. 516–17).

Writing taxonomic names – always underline (handwritten text) or italicise (word-processed text) generic and species names to avoid confusion: thus *bacillus* is a descriptive term for rod-shaped bacteria, while *Bacillus* is a generic name.

Six other levels of taxa are generally accepted: division/phylum, class, order, family, genus and species.

KEY POINT Whereas the levels of taxa above genus are rather subjective and vary among authorities, the use of genus and species names are governed by strict, internationally agreed conventions called Codes of Nomenclature.

There are three major Codes of Nomenclature for cellular organisms, the Prokaryote and Zoological Codes plus one for Algae, Fungi and Plants (formerly the Botanical Code), which operate on similar but not identical principles (Ride *et al.*, 1999; Turland *et al.*, 2018; Parker *et al.*, 2019).

Understanding the basis of classification

No simple definition of a species is possible, but there are two generally used definitions:

1. **A group of organisms capable of interbreeding and producing fertile offspring** – this, however, excludes all asexual, parthenogenetic and apomictic forms.
2. **A group of organisms showing a close similarity in phenotypic characteristics** – this would include morphological, anatomical, biochemical, ecological and life history characters.

KEY POINT The basic unit of classification is the species, which represents a group of recognisably similar individuals, clearly distinct from other such groups.

When species are compared, groups of species may show a number of features in common; they are then arranged into larger groupings known as genera (singular genus). This process can be repeated at each taxonomic stage to form a hierarchical system of classification whose different levels are known as taxonomic ranks. The number of levels in this system is arbitrary and based upon practical experience – the seven levels normally used have been found to be sufficient to accommodate the majority of the variation observed in nature.

When a generic or specific name is changed as a result of further study, the former name becomes a synonym; you should always try to use the latest name. Where a generic name has been changed recently, the old name is occasionally given in parentheses to allow easy reference to the old name.

Naming organisms

The scientific name of an organism is effectively a symbol or cipher that removes the need for repeated use of descriptions. It normally comprises two words and is, therefore, called a binomial term; for example, *Escherichia coli*. The name of the genus is followed by a second term that identifies the species, for example, *Pseudomonas aeruginosa* or *Saccharomyces cerevisiae* (Table 37.1).

The Codes of Nomenclature were established to prevent the ambiguities associated with informal/common names. The Codes require that all scientific names are either Latin or treated as Latin, written in the Latin alphabet and subject to the rules of Latin grammar. Consequently, you must be very precise in your use of such names. In some cases, the Codes

Table 37.1 Example of taxonomies for a bacterium and a fungus.

Common name	<i>Pseudomonas</i>	Baker's yeast
Kingdom	Monera	Fungi
Division/Phylum	Gracilicutes	Ascomycota
Class	Scotobacteria	Incertae sedis ¹
Order	Pseudomonadales	Saccharomycetales
Family	Pseudomonadaceae	Saccharomycetaceae
Genus	<i>Pseudomonas</i>	<i>Saccharomyces</i>
Species	<i>P. aeruginosa</i>	<i>S. cerevisiae</i>

¹ Meaning = 'uncertain taxonomic position'.

Example The full, formal name of baker's yeast is *Saccharomyces cerevisiae* Meyen ex Hansen 1883. After first use, this would be abbreviated to *S. cerevisiae*.

stipulate a standardised ending for the names of all taxa of a given taxonomic rank, for example, fungal and bacterial families end in -aceae. When used in a formal scientific context, you should follow the specific name by the authority on which that name is based, i.e. the name of the person describing that species and the date of the description.

Box 37.1 summarises the basic rules for writing taxonomic names.

Box 37.1 How to record taxonomic names

- Names of the seven levels of taxa should take lowercase initial letters, e.g. kingdom Fungi.
- The Latin forms of all taxon names except the specific name take initial capital letters, e.g. 'the Ascomycota ...' but anglicised versions do not, e.g. 'the ascomycetes...'
- The names of the higher taxa are all plural, hence 'the Ascomycota are...' while the singular of the anglicised version is used for a single member of that taxon, hence 'an ascomycete is...'
- The binomial system gives each species two names, the first being the generic name and the second the specific name, which must never be used by itself. The genus and species names are distinguished from the rest of the text either:
 - by being underlined (when handwritten), e.g. Penicillium notatum; or
 - by being set in italics (in print or on a word processor), e.g. *Penicillium notatum*.
- The generic name is singular and always takes an initial capital letter. If you use the generic name this implies that the point being made is a generic characteristic unless the specific name is present. Write the generic name in full when first used in a text, e.g. *Escherichia coli*, but subsequent references can be abbreviated to its initial letter, e.g. *E. coli*, unless this will result in confusion with another genus also being considered; for example, with *Escherichia coli* and *Enterococcus faecalis*, these could be abbreviated to *Esch. coli* and *Ent. faecalis*, to avoid any confusion.
- The abbreviation 'sp.' should be used in place of the specific name if a single unspecified species of a genus is being referred to, e.g. *Bacillus* sp.; it is not underlined or italicised. If more than one unspecified species is meant, then the correct form is 'spp.', e.g. *Bacillus* spp.
- The name of each species should be followed by the authority: on first usage in formal reports and in titles, the name or names of the person(s) to whom that name is attributed and the date of that description should be quoted. These names may sometimes be abbreviated. If the species was first described under its current generic name, the authority's name, often in abbreviated form, is added. If, however, the species was first described under a different genus, the name of the author of the original description is presented in parentheses, e.g. *Escherichia coli* (Migula) Castellani and Chalmers. The use of authorities should be confined to formal papers, final-year project reports, etc.; they would not normally be used in practical reports, short assignments or examinations.

Applying bacterial typing methods – typing methods are used widely in epidemiological studies, e.g. tracing a particular type of bacterium responsible for a food-poisoning outbreak, or tracking the development of a particular type of antibiotic-resistant bacterium in a hospital.

Understanding microbiological terminology – the term *strain* is widely used, particularly in the context of the practice of lodging microbiological strains with culture collections, while the term *isolate* is often used for a pure culture derived from a natural (wild) population. Cell lines (Chapter 41) are often given code names and/or reference numbers.

Naming animal and human viruses and the diseases they cause – historically, these were often named after the scientist who first described the condition (example: Marburg disease/virus), or by location (examples: Ebola and Hendra disease/virus). However, since 2015, WHO guidelines recommend more descriptive terms (example: the coronavirus disease identified in 2019 (**COVID-19**) is caused by the virus SARS-CoV-2).

Naming taxa below the rank of species

In microbial classification, use is often made of taxa below the rank of species.

In bacteriology, the use of subspecies is acceptable although a word indicating rank is usually inserted, for example, *Bacillus subtilis* subsp. *niger*. However, other terms are in widespread use for taxa below the species level, especially in medical microbiology and plant pathology, when a particular strain of bacterium has been identified (p. 260). Subspecies identification is often referred to as typing and the following terms apply:

- **biovar, or biotype:** subdivided according to biochemical characteristics
- **serovar or serotype:** subdivided by serological methods, using antibodies (see Chapter 53)
- **pathovar:** subdivided according to pathogenicity (ability to cause disease)
- **phagovar or phage type:** subdivided according to susceptibility to particular viruses.

Many micro-organisms are now referred to by their generic and specific names followed by a culture collection reference number, for example, *Bacillus subtilis* NCTC 10400, where NCTC stands for the National Collection of Type Cultures and 10400 is the reference number of that strain in the collection.

Naming viruses

The classification and nomenclature of viruses are less advanced than for cellular organisms and the current nomenclature has been arrived at on a piecemeal, *ad hoc* basis. The International Committee for Virus Taxonomy proposed a unified classification system, dividing viruses into 50 families on the basis of:

- **host preference**
- **nucleic acid type** (i.e. DNA or RNA)
- **whether the nucleic acid is single- or double-stranded**
- **the presence or absence of a surrounding envelope.**

Virus family names end in *viridae* and genus names in *virus*. (Note that these names are *not* latinised and the genus-species binomial is not now approved.) However, this system has not yet been adopted universally and many viruses are still referred to by their trivial names or by code names (sigla), for example, the bacterial viruses fX174, T4, etc. Many of the names used reflect the diseases caused by the virus. Often, a three-letter abbreviation is used, for example, HIV (for human immunodeficiency virus), TMV (for tobacco mosaic virus). 'A virus obtained from a single host and replicated in the laboratory is termed an 'isolate' and is typically referred to in terms of isolate number and year of isolation. In contrast, a virus with a genomic sequence different to the reference sequence is termed a 'variant' – for example, the variants of SARS-CoV-2 identified in different countries during the COVID-19 pandemic.'

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STUDY EXERCISES

37.1 What is wrong with the hierarchical classifications given in the table below?

Examples of hierarchical classifications

<i>Nautilus</i>	Edible mushroom	<i>E. coli</i> bacterium
Animalia	Fungi	Bacteria (Monera)
Mollusca	Basidiomycota	Proteobacteria
Cephalopoda	Basidiomycetes	Enterobacteriales
Nautilidae	Agaricales	Gamma subdivision
Nautilida	Agaricaceae	Enterobacteriaceae
<i>Nautilus</i>	<i>Agaricus</i>	<i>Escherichia</i>
<i>pompilius</i>	<i>bisporus</i>	<i>coli</i>

37.2 Compare a variety of current textbooks and Internet sites to discover alternative classification schemes at kingdom level. Make your own notes regarding the *evidence* used to support the alternative kingdom classifications.

37.3 Research full classifications. Provide the full classification of the following species, laid out as in the table in Study exercise 37.2.

- The limpet *Patella vulgata*
- The great white shark
- The giant redwood
- The earthworm *Lumbricus terrestris*.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

38 Working with bacteria

Understanding Archaea – these microbes are named from the Greek ‘arkhaia’ (ancient), due to their ancient separation from bacteria, together with the ‘extremophile’ features of many early isolates that may resemble growth conditions similar to those of primitive Earth. However, they are also found in a wide range of non-extreme environments, including soil and water.

Definitions

Pathogen – an organism that causes disease (*pathology-generating*).

Peptidoglycan – found only in bacteria, comprising unique monosaccharide and amino acid subunits. Peptidoglycan is digested by lysozyme – part of the mammalian innate immune system.

Teichoic acids – polymers of polyol phosphates linked to sugar and/or amino acid residues. Contributing to the net negative charge of the Gram-positive cell wall.

Lipopolysaccharide (LPS) – present in the exterior-facing layer of the outer membrane of Gram-negative bacteria, comprising characteristic sugar and lipid subunits. Also known as ‘endotoxin’ and ‘O antigen’, triggering mammalian immune response systems.

Bacteria (formerly termed Eubacteria) are prokaryotic microbes, lacking a membrane-bound nucleus (Chapter 37) and growing by binary fission. Comparative rRNA sequencing (Fig. 37.1) first differentiated them from the other major domain of prokaryotes, the Archaea (formerly termed Archaeobacteria); subsequent studies have shown fundamental differences in membrane lipid and cell wall composition between the two groups.

The bacterial domain encompasses organisms that differ in their requirements for carbon and energy (Chapter 34), including heterotrophs (for example, bacteria that grow on eukaryotic organisms), photoautotrophs (for example, cyanobacteria) and chemoautotrophs (for example, sulfur-oxidising bacteria). Heterotrophic bacteria are the most studied group, since this includes all pathogenic (disease-causing) forms, together with opportunistic pathogens and non-pathogenic forms. This chapter focuses on culturing heterotrophic bacteria, as you are most likely to use these microbes during your practical work. Specialist texts should be consulted for other groups – for example, Mishra *et al.* (2018) for cyanobacteria. Chapter 67–69 cover the basic methodology for molecular genetics procedures.

An important practical characteristic of different groups of heterotrophic bacteria is their Gram status – based on their reaction to the Gram staining procedure (Box 36.1 p. 259)

- 1. Gram-positive bacteria:** these have a thick layer of the heteropolymer peptidoglycan within their cell walls, complexed with teichoic acids, often with an additional outer S layer (S = surface) composed of protein (Fig. 38.1(a)). They include the endospore-forming genera *Bacillus* (aerobe) and *Clostridium* (anaerobe).
- 2. Gram-negative bacteria:** these have a thin layer of peptidoglycan, plus an outer membrane containing lipopolysaccharide; the outer membrane surrounds the peptidoglycan layer, creating a periplasmic region between the plasma membrane and the outer membrane (Fig 38.1(b)). They include *Escherichia coli* (*E. coli*), *Salmonella typhi* and *Vibrio cholerae*.

Some other groups of bacteria, including the mycoplasmas, have no cells walls; these wall-less forms retain little colour when Gram stained, and are best visualised using a DNA-binding fluorescent stain such as 4',6-diamidino-2-phenylindole (DAPI).

Working safely with bacteria

The importance of safe working with all bacterial samples cannot be overstated. Chapter 33 covers the general principles, including the classification of microbes into different hazard groups, based on their virulence (severity of disease) and transmissibility (Table 47.1). Many routine lab classes are carried out using ACPD Hazard Group 1 bacteria (those unlikely to be pathogenic to humans), to minimize risk. However, other sessions – for example, environmental microbiology classes may use uncharacterised isolates, while medical bacteriology classes may use microbes from ACPD Hazard Group 2. All microbes from ACDP Hazard Groups 3 and 4 must be handled in dedicated biosafety cabinets, following strict protocols to prevent infection.

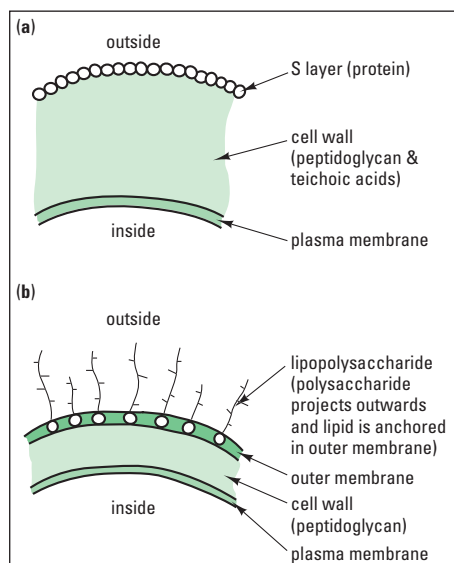


Fig. 38.1 Cell envelopes of (a) Gram-positive and (b) Gram-negative bacteria.

Definition

Type culture – a standard of reference strain of known origin, maintained in a recognised culture collection.

Examples Formulae for standard bacterial culture media:

Nutrient broth typically contains: 0.2% w/v yeast extract, 0.5% w/v peptone and 0.5% w/v NaCl, pH 6.8–7.4.

Nutrient agar typically contains an additional 1.5% w/v agar.

KEY POINT Irrespective of the types of bacteria you handle in lab classes, you must *always* follow good practice in sterile technique, minimising the likelihood of inhalation, ingestion or inoculation (Fig. 20.1, p. 127), alongside any specific guidance resulting from the risk assessment for the procedure.

Selecting growth conditions

The two main types of bacteria used for laboratory culture are:

- **Strains** – derived from known organisms, typically held a national culture collection (for example, the UK National Collection of Type Cultures, NCTC). Such strains are of defined provenance and their growth requirements are mostly well characterised.
- **Isolates** – for example, those obtained directly from food, soil or water during a laboratory exercise. The optimum growth requirements and potential pathogenicity of such isolates will be unknown – often, repeated sub-culture in the laboratory will ‘train’ isolates to grow well in the medium use for sub-culture. This phenomenon is also seen when strains are transferred from one type of growth medium to another of different composition.

Choosing an appropriate culture medium

Most of the bacteriological media that you will use are based on complex (undefined) natural ingredients, which include meat/beef extract and/or yeast extract, together with hydrolysed protein (peptone – a pepsin-based digest of animal tissue), typically at near neutral pH. Using such media will provide a wide range of nutrients that enable the growth of most non-fastidious heterotrophs such as *E. coli*. Most media are available in liquid (broth) and solidified (agar-based) formats, depending on whether your aim is to grow the bacteria as cell suspensions (broth) or colonies (agar medium). Your alternative approach is to use a defined (synthetic) medium, which uses only chemically specified constituents – an example is BG-11 medium (Rippka *et al.*, 1979), which uses several inorganic salts and trace metals for the growth of photoautotrophic cyanobacteria.

When culturing fastidious heterotrophic bacteria you may need to include additional constituents to enable them to be grown in the laboratory: for example, blood is often added to enrichment media for human pathogenic bacteria. You can use blood agar to detect the destruction of red cells by haemolytic bacteria such as *Streptococcus pyogenes*, which completely breaks down red cells to give a clear zone of ‘beta’ haemolysis around each colony on blood agar, whereas others, such as *Streptococcus pneumoniae*, shows only partial breakdown of red cells, giving a green zone of ‘alpha’ haemolysis. In contrast, the obligate human pathogen *Neisseria gonorrhoeae*, requires a medium enriched with lysed blood cells in order to grow, since it requires the nicotinamide adenine dinucleotide (NAD) co-factor released by lysis of red cells.

When culturing a particular group of bacteria, you may need to select a medium with specific additional ingredients, designed to provide selective and diagnostic characteristics for that group. Table 34.1 gives details of the range of selective agents used in routine media. Differential media, also known as diagnostic media, distinguish between different sub-groups of bacteria growing on the same medium. One example is blood agar, which

Examples Formulae for specialised bacterial culture media:

Blood agar typically contains 5% v/v sheep blood in addition to peptone and NaCl – the blood is added once the autoclaved medium has cooled to $\cong 50^\circ\text{C}$, to prevent lysis of red cells.

Chocolate agar, so named because of its mid-brown colour, contains heat-treated (lysed) 5% w/v sheep blood – used for fastidious pathogens such as *Neisseria* spp. and *Haemophilus* spp.

distinguishes between (i) beta haemolytic (ii) alpha haemolytic and (iii) non-haemolytic bacteria. Other differential media contain nutrients that only some sub-groups of bacteria can use, often leading to a colour change, due either to a pH indicator dye or a chromogenic substrate that detects a particular enzyme found in the target group (Chapter 62). Box 38.1 gives details for MacConkey medium, a selective, differential medium used in clinical bacteriology for the isolation and characterisation of certain faecal bacteria, depending on their ability to metabolise the disaccharide lactose.

Deciding on a suitable growth temperature

Most commensal and pathogenic bacteria isolated from humans and mammals are mesophiles (p. 253), growing well in an incubator at 37°C (human body temperature) while environmental organisms are more often grown at $20\text{--}25^\circ\text{C}$. Dedicated incubators are required for thermophiles ($>45^\circ\text{C}$) or psychrophiles ($<20^\circ\text{C}$). Under conditions where the door of an incubator is repeatedly opened and closed, the temperature inside can fluctuate markedly – consider whether to keep temperature-sensitive cultures within a box inside such an incubator, you should minimise their exposure to temperature fluctuations.

Selecting a suitable atmosphere

While many routine cultures are grown in the unmodified atmosphere of the laboratory, some bacteria require more controlled conditions. As examples of the former, many heterotrophic bacteria are facultative anaerobes (p. 255); consequently, you can grow cultures of this group of bacteria in an aerobic incubator, which you can also use for growing obligate aerobes

Box 38.1 How to use a differential medium for bacterial isolation: an example

MacConkey agar is both a selective and a differential medium, useful for the isolation and identification of intestinal Gram-negative bacteria. You should understand that each component in the medium has a particular role:

- **Peptone:** (a meat digest) provides a rich source of complex organic nutrients, to support the growth of non-exacting bacteria.
- **Bile salts:** toxic to most microbes apart from those growing in the intestinal tract (selective agent).
- **Lactose:** present as an additional, specific carbon source (enrichment agent).
- **Neutral red:** a pH indicator dye, to show the decrease in pH that accompanies the breakdown of lactose.
- **Crystal violet:** selectively inhibits the growth of Gram-positive bacteria. (This is only present in certain formulations of MacConkey agar.)

Interpreting growth on MacConkey agar

You can distinguish between two main outcomes using this medium:

1. Intestinal Gram-negative bacteria capable of fermenting lactose will grow to produce red-purple colonies, the red coloration being due to the neutral red indicator under low pH conditions (acidic breakdown product of lactose metabolism), while the purple coloration, often accompanied by a metallic sheen, is due to the precipitation of bile salts and crystal violet at low pH.
2. Intestinal Gram-negative bacteria unable to metabolise lactose will give colonies with no obvious pigmentation.

This differential medium is particularly useful in medical microbiology, since many enteric bacteria are unable to ferment lactose (e.g. *Salmonella*, *Shigella*) while others metabolise this carbohydrate (e.g. *E. coli*, *Klebsiella* spp.). You can also investigate different colonial morphologies (Fig. 39.2) using this medium: for example, capsulate *Klebsiella* spp. characteristically produce large, convex, mucoid colonies with a weak pink coloration, due to the fermentation of lactose, while *E. coli* produces smaller, flattened colonies with a stronger red coloration and a metallic sheen.

Culturing injured and stressed bacteria – sub-lethal injury can occur during the process of isolating bacteria from the environment (Chapter 35). Injured cells are often more sensitive to oxygen and oxygen free radicals (e.g. singlet oxygen and peroxides) than healthy cells, requiring additives such as pyruvate and catalase to be added to growth media for their successful cultivation. Such cells also may be inhibited by the agents used in selective isolation media (Box 38.1).

Growing stressed and injured bacteria – these will often grow more readily in broth culture than at the surface of an agar medium, since the oxygen level is lower and oxygen toxicity is reduced.

and aerotolerant anaerobes (p. 255). However, to culture strict anaerobes you will require an oxygen-free environment, since they may be killed by even a short exposure to atmospheric oxygen. To achieve this, you can use either an anaerobic jar (Fig. 38.2) or a dedicated anaerobic cabinet. Capnophilic bacteria such as *Campylobacter* spp. grow best under condition of high CO₂, typically \cong 5% v/v, and low O₂, typically <5% v/v. This is best achieved using a sealed jar or an incubator whose gas composition can be controlled.

Setting up agar-based cultures

Many culture methods make use of a growth medium solidified with agar within a Petri plate (= Petri dish). You can use a variety of techniques to transfer and distribute the organisms prior to incubation, with the three most important procedures described in Box 38.2.

Preparing broth-based cultures

Growing bacteria as a cell suspension in broth has several advantages, including:

- **the suspension consists of cells of broadly similar age**, in contrast to colonies on agar-based media, where the outermost edge of the colony contains active cells while cells towards the centre of the colony will be older and may be senescent
- **large amounts of active, growing cells can be produced**
- **the dynamics of population growth can be studied** (p. 242)
- **continuous cultures can be established** (pp. 243–4).

Disadvantages of broth culture include:

- **It can be difficult to detect contamination**, since all bacterial growth appears similarly turbid in liquid culture. To check for contamination, you should prepare streak dilutions of the broth and look for colonies with different morphology (size, shape, colour) to those of the cultured bacterium.
- **Broth cultures lack the colony characteristics seen with agar-based cultures, limiting their use in conventional identification schemes** (Chapter 36).
- **Cloning procedures (p. 529) are easiest to perform using colonies on agar-based media**, since all cells in a colony will be clones of the original inoculum.

You should consider the advantages and disadvantages of each method when choosing between broth and agar-based culture. Consult the literature before setting out on advanced practical and project work, to learn from others who have optimised the growth conditions for a particular target microbe.

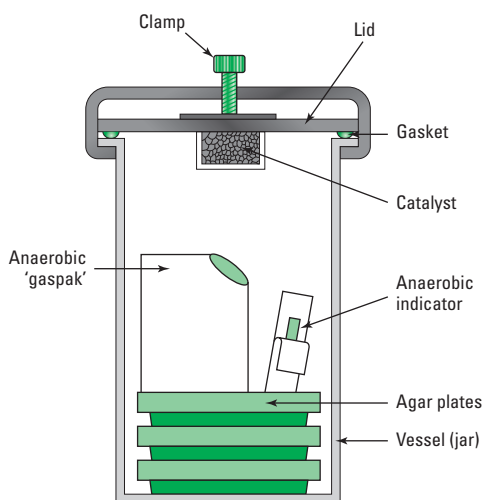


Fig. 38.2 An anaerobic jar.

Box 38.2 How to prepare agar-based cultures of bacteria

The main procedures used with agar-based media are:

A Streak-dilution plating

Preparing a streak plate for single colonies is one of the most important basic skills in microbiology, since it is used in the initial isolation of a cell culture and in maintaining stock cultures of bacteria and yeasts, where a streak-dilution plate with single colonies all of the same type confirms the purity of the strain (colonies of contaminant microbes typically appear different in size, colour or form). A sterile inoculating loop (p. 237) is used to streak the sample over the surface of the medium, thereby diluting any bacteria present in the sample. The aim is to achieve single colonies at some point on the plate: ideally, such colonies are derived from single cells (e.g. in the case of bacteria with a unicellular growth habit) or from groups of cells of the same species (e.g. in filamentous or colonial forms), which forms that basis of most pure culture methods.

Carry out streak dilution as follows:

- **Using a sterile inoculating loop, take a small sample of the material to be streaked.** Distribute the sample over a small sector of the plate (area 1, Fig. 38.3(a)), then flame the loop and allow to cool (approximately 8–10 s).

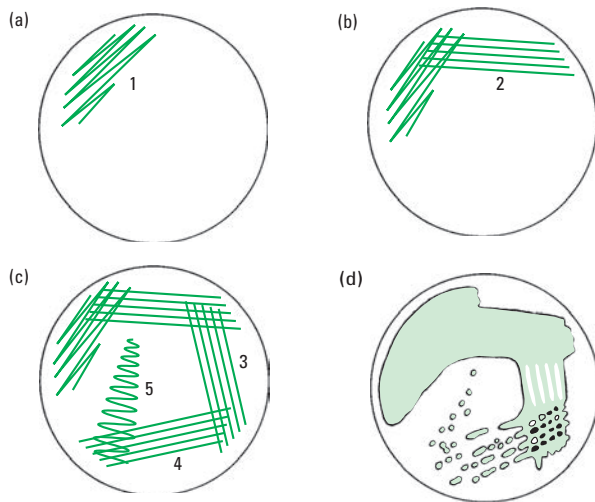


Fig. 38.3 Stages in preparing a streak dilution plate.

- **Make several small streaks from the initial sector into the adjacent sector** (area 2, Fig. 38.3(b)), taking care not to allow the streaks to overlap. Flame the loop and allow to cool.
- **Repeat the procedure for the adjacent areas of the plate** (areas 3 and 4, Fig. 38.3(c)), re-sterilising the loop between

each step. End with a single, long streak in the remaining sector of the plate, as shown (area 5, Fig. 38.3(c)).

- **Examine after incubation at the appropriate temperature** (e.g. 37 °C for 24 h): each step should have diluted the inoculum, giving individual colonies within one or more sectors (Fig. 38.3(d)). Further subculture of an individual colony should give a pure (clonal) culture.

Note the following:

- **Keep the lid of the Petri plate as close to the base as possible** during the streaking procedure, to reduce the risk of aerial contamination. Working within the 'updraft zone' close to a Bunsen burner (Fig. 47.2) can also minimise the chance of airborne contaminants.
- **Allow the loop to glide over the surface of the medium.** Hold the handle near the balance point and use light, sweeping movements, as the agar surface is easily damaged and torn.
- **Work quickly, but carefully.** Do not breathe directly over the exposed agar surface and replace the lid as soon as possible.

B Spread plating

This method is often used with cells in suspension – either in a liquid growth medium (broth) or in an appropriate sterile diluent. It is one method of quantifying the number of viable cells (strictly, colony-forming units, CFU) in a sample, after appropriate dilution. Box 34.2 gives details of the quantitative procedure. Prepare a spread plate as follows:

- **Transfer a small volume of cell suspension** (0.05–0.5 mL) to the surface of a solidified medium in a Petri plate (Fig. 38.4(a)).
- **Sterilise an L-shaped glass spreader** by dipping the end in a beaker containing a small amount of 70% v/v alcohol, allowing the excess to drain from the spreader and then igniting the residual alcohol in a Bunsen flame (Fig. 38.4(b)). Allow to cool (8–10 s).
- **Distribute the liquid over the surface of the plate using the sterile spreader.** Ensure an even coverage by rotating the plate as you spread (Fig. 38.4(c)): allow the liquid to be absorbed into the agar medium.
- **Examine after incubation under suitable conditions.** The microbial colonies should be distributed evenly across the surface of the plate.

Note that there is a significant fire hazard associated with this version of spread plating, so take care not to ignite the

(continued)

Box 38.2 (continued)

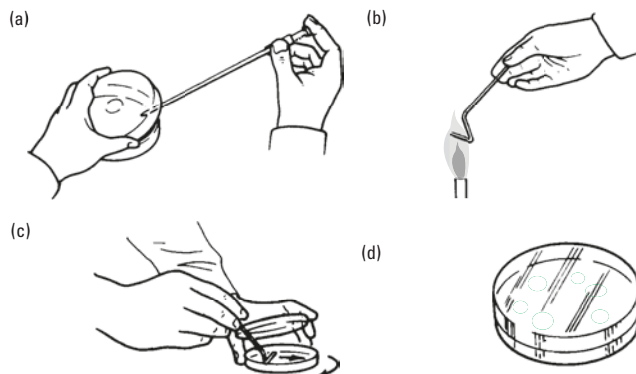


Fig. 38.4 Stages in preparing a spread plate.

alcohol in the beaker, e.g. by returning an overheated glass rod to the beaker. The alcohol will burn with a pale blue flame that may be difficult to see, but will readily ignite other materials (e.g. a laboratory coat). Another source of fire hazard comes from small droplets of flaming alcohol shed by an overloaded spreader on to the bench and this is why you *must* drain excess alcohol from the spreader *before* flaming. Some laboratories now provide plastic disposable spreaders for student use, to avoid this hazard.

C Pour plating

This procedure also uses cells in suspension, as for spread plating. However, pour plating requires *molten* agar medium. Prepare a pour plate as follows:

- **Prepare aliquots of molten agar** – these are usually in screw-capped bottles containing sufficient medium to prepare a single Petri plate (i.e. 15–20 mL), kept in a water bath at 45–50 °C until required.
- **Add a known volume of cell suspension** (0.05–1.0 mL) to an individual bottle of molten agar medium (Fig. 38.5(a)).
- **Mix thoroughly, by rotating between the palms of the hands** (Fig 38.5(b)): do not shake or this will cause frothing of the medium. Work without delay, or the molten medium will set inside the bottle.
- **Pour all of the mixture into an empty, sterile Petri plate** (Fig. 38.5(c)). Allow to set. Most microbes are not killed by brief exposure to temperatures of 45–50 °C, though

the procedure may be more damaging to those from low temperature environments, for example, psychrophilic bacteria.

- **Examine after incubation under suitable conditions.** The colonies will be distributed throughout the medium: any cells deposited at the surface will give larger, spreading colonies (Fig. 38.5(d)). As most of the colonies are formed within the medium, they are far smaller than those of the surface streak method, allowing higher cell numbers to be counted (e.g. up to 1000 colonies per pour plate, rather than up to 30 colonies per streak dilution plate). One further modification used by some workers is to pour a thin layer of molten agar on to the surface of a pour plate after it has set, to ensure that no surface colonies are produced.

Note that a disadvantage of the pour-plate method is that the typical colony morphology seen in surface-grown cultures will not be observed for those colonies that develop within the agar medium. A further disadvantage is that some of the suspension will be left behind in the screw-capped bottle. You can avoid the latter by transferring your cell suspension to the Petri plate, adding the molten agar, then swirling the plate gently to mix the two liquids within the plate. However, even when the plate is swirled repeatedly and in several directions, the liquids are never mixed as evenly as in the former procedure.

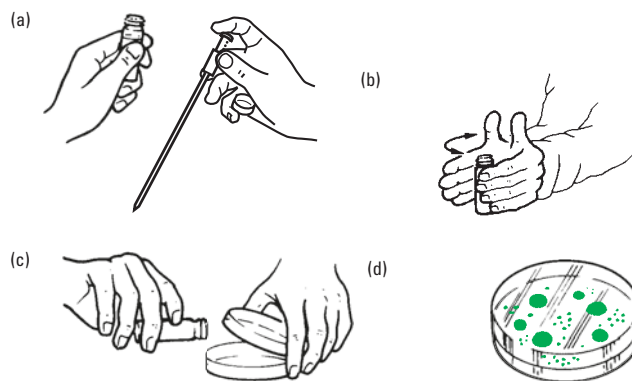


Fig. 38.5 Stages in preparing a pour plate.

Harvesting cultures

You can harvest bacteria and yeast from the surface of agar-based media by scraping colonies using a sterile inoculating loop or a scalpel. A few drops of sterile saline can be used to help bring cells into suspension – mix colonies with the saline and then remove using a sterile Pasteur pipette

(p. 143). Broth cultures can be harvested either by filtration (typically, for small amounts) or centrifugation (Chapter 44). You can then use harvested cells for other practical procedures – for example, physiological experiments such as measurement of respiration, or studies of respiratory inhibitors (Chapter 64).

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STUDY EXERCISES

38.1 Investigate the growth requirements of specific bacteria. How might you culture the following bacteria in the laboratory?

- (a) *Bacillus cereus*
- (b) *Clostridium perfringens*
- (c) *Vibrio cholerae*
- (d) *Neisseria meningitidis*

38.2 Investigate the controversy around so-called 'viable but non-culturable' bacteria. Carry out a

Google Scholar search, or equivalent, and list some of the arguments for and against the idea that some viable bacteria may enter a non-culturable state. Are you convinced by one side or the other?

38.3 Consider the advantages and disadvantages of spread-plating and pour-plating methods. Having read through this chapter, list up to six pros/cons of each plating method and compare your answers either with the list that we have provided on the website, or with those of other students as a group exercise.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

39 Working with eukaryotic microbes: fungi and protists

Eukaryotic microbes are characterised by their microscopic dimensions, together with the presence of a membrane-bound nucleus and organelles. As such, they are a heterogeneous group that can be sub-divided in many different ways. This chapter focusses on fungi (eukaryotic, walled heterotrophs) and protists (a broad kingdom that includes protozoa and microalgae as two major sub-groups, pp. 277–8). Practical techniques for laboratory culture and microscopy are emphasised, focussing on procedures you are most likely to use in lab classes. For molecular methods, see Chapter 67–69.

KEY POINT The combination of cell walls and heterotrophic nutrition characterises fungi as members of a unique kingdom, distinct from animals, plants and other eukaryotes. Protists, on the other hand, comprise those eukaryotic organisms that are not regarded as plants, animals or fungi, being defined by what they are not.

Understanding fungal diversity and terminology

Fungi are characterised by the following combination of features:

- **Cell structure.** As eukaryotes, fungal cells contain nuclei and other membrane-bound organelles (Fig. 39.1).
- **Cell wall.** Typically present and composed of chitin or glucans (rarely, cellulose).
- **Nutrition.** Fungi obtain carbon and energy heterotrophically, by the production of exoenzymes and absorption of nutrients from the surrounding environment, rather than by ingestion and digestion as in most animals.
- **Reproduction.** May include asexual and sexual cycles. Reproduction typically involves the production of single-celled spores that are able to survive adverse environmental conditions, to enable colonisation of new habitats, typically by dispersal in air, being resistant to desiccation and sunlight. Fungi can be further sub-divided on the basis of the different spore types – for example, ascomycete fungi are characterised by their ascospores, produced in a sac-like structure (ascus, from Greek *askos* = sack).

Fungi are also differentiated on the basis of their growth form. Typically, three broad groups are recognised:

1. **Unicellular fungi (yeasts).** Growing predominantly as single cells, they are usually sub-divided on the basis of their means of cell proliferation, with budding yeasts producing daughter cells that grow out from a ‘bud’ produced on the parent cell (Fig. 39.1), while fission yeasts multiply by binary fission, enlarging and then dividing in two.
2. **Filamentous fungi (moulds).** Individual organisms grow as a spreading network of branched walled filaments filled with cytoplasm; these filaments are termed hyphae (singular: hypha). The entire network

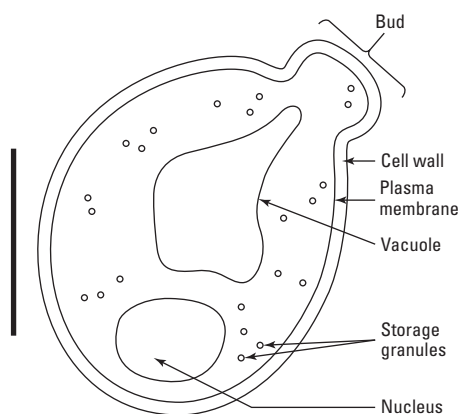


Fig. 39.1 Fungal cell structure: *Saccharomyces* sp. (budding yeast). Storage granules are protein, found in quiescent cells. The bud will develop into a new cell, which will then break free of the parent cell. Scale bar = 5 μ m.

SAFETY NOTE – while fungal activity is associated with a characteristic musty odour (e.g. in soils, p. 258) you must take great care to avoid inhaling spores when working with fungi in the laboratory: keep lids on Petri plates and culture tubes and avoid creating aerosols (p. 239)

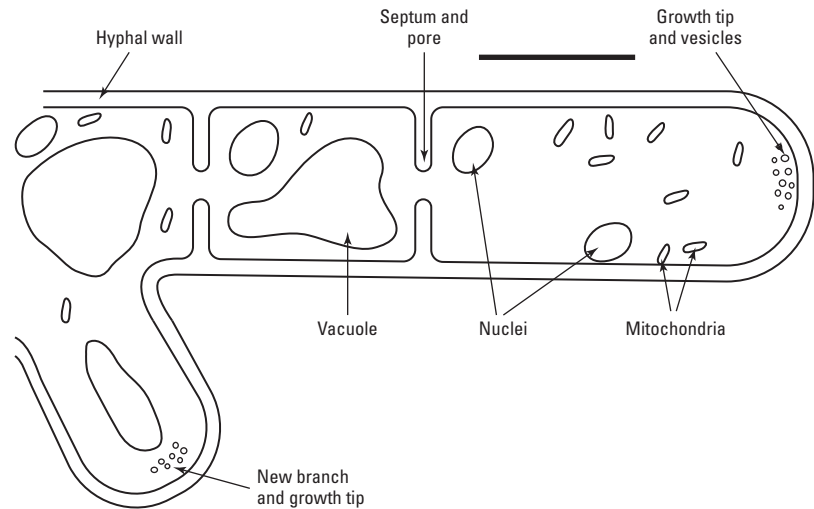


Fig. 39.2 Structure of a typical fungal hypha. Vesicles at the hyphal tips are involved in growth (hyphal extension). Scale bar = 10 μm .

Understanding the genetic status of filamentous fungi – mycelia can be derived from a single spore, with all nuclei being genetically identical (homokaryon) or by fusion (anastomosis) of genetically different hyphae (heterokaryon).

is described as a mycelium (plural mycelia). Figure 39.2 shows the structure of a typical hypha. Fungal mycelia can be very large – probably the largest organisms on Earth (Schmitt and Tatum, 2009). However, while the length and branching of the network is indeterminate, growing from the tips of each hypha, the diameter of an individual hypha is microscopic, (typically 4–30 μm), hence their classification as microbes. While hyphae often have cross-walls (septa), they are not typically organised into individual cells, but have nuclei within a common cytoplasm (coenocytic structure, Fig 39.2).

- 3. Dimorphic fungi.** Some fungi can grow as either filamentous hyphae or single cells (dimorphic = two forms), often switching between the two depending on environmental conditions. For example, the dimorphic fungus *Candida albicans* is typically a non-pathogenic commensal on human mucosal surfaces in its yeast-like form, becoming invasive in its filamentous form and causing diseases such as thrush. As a practical exercise, this transition can be triggered in the laboratory by growth temperature, with yeast-like growth of *C. albicans* being favoured at 25 °C and filamentous growth at 37 °C. Other dimorphic fungi show the opposite response to temperature ('mould in the cold, yeast in the heat', for example, the human pathogens *Histoplasma capsulatum* and *Penicillium marneffei*).

Definition

Polyphyletic group – one having no immediate shared common evolutionary ancestor, being grouped together on the basis of the presence or absence of certain characteristics, rather than evolutionary relationships (phylogenetic taxonomy, p. 264).

Understanding protist diversity and terminology

This polyphyletic group shares a few common characteristics, including:

- **Cell structure.** This always involves a membrane bound nucleus and cytoplasmic organelles.
- **Organisation.** Typically, most grow as individual cells (unicellular forms). Some cells may form filaments or clumps/colonies. However, they do not show the tissue differentiation seen in animals and plants.
- **Reproduction.** Most protists reproduce by asexual means, typically by binary fission.

Understanding the life cycles of parasitic protozoa – these are often complex, with different feeding stages (trophozoites) in one or more hosts. Consult a specialist text (e.g. Florin-Christensen and Schnitger, 2018) for detail of terminology of different stages.

Working with microalgae – some microalgae produce toxins, with the dinoflagellates being among the most toxic. Avoid skin contact or ingestion.

Studying fungal morphology – the following are representative practical experiments (British Mycological Society, 2020):

- **Observing spores:** obtain basidiospores from a sample of a mature mushroom cap. Mount these in water and observe using oil immersion (x100 objective, Chapter 26). By sampling from a known area of gill, using a known volume of water, estimating the total area of gills, and scaling up, it is possible to estimate the numbers of spores produced per mm² of gill and then of the whole reproductive structure.
- **Observing growing hyphae:** sterilised plant seeds (e.g. hemp) are placed in oxygenated pond water for a week, then the communities of fungi (and other microorganisms) feeding on each seed can be observed using light microscopy. The x40 objective lens should provide sufficient magnification for these studies.

Protists are often further divided into two broad groups on the basis of their nutrition:

1. **Protozoa.** Typically feed by heterotrophy and lack a cell wall (historically viewed as single-celled animals). Some protozoa are able to form cysts that resist environmental extremes.
2. **Microalgae.** Typically obtain their carbon and energy via photosynthesis (historically regarded as single-celled plants). Forming a large component of the plankton of both freshwater and seawater, they are distinguished from the photosynthetic cyanobacteria (formerly termed blue-green algae) by their eukaryotic cell structure. Some microalgae are facultative heterotrophs and are capable of using complex organic molecules as a source of carbon and energy. Microalgae such as *Chlorella* are often used as model organisms to study physiological processes such as photosynthesis, using an oxygen electrode (Chapter 64).

Investigating eukaryotic microbes using light microscopy

Fungi

Direct microscopy can be useful starting point for characterising fungi in environmental samples (for example, soil fungi, or pathogens of humans, animals and plants) and in laboratory culture. Typically, lactophenol cotton blue (LCB) is used as a mountant – cotton (methyl) blue is a preferential stain for the chitin present in fungal cell walls while lactophenol (an aqueous mixture of lactic acid, phenol and glycerol) acts as a fixative and preservative. Box 39.1 gives details of the procedure. Alternative stains are sometimes used to detect fungi in mammalian tissue samples, including silver stains and periodic acid-Schiff reagent, while a combination of safranin and cotton blue can differentiate plant tissues (red) from pathogenic fungi (blue).

The asexual and sexual spores produced by different fungi are often characteristic of a particular group and are used as an aid to identification. As a consequence, different terms have been created for the various types of spores. Thus the products of budding (asexual reproduction, Fig. 39.1) in yeasts such as *Saccharomyces cerevisiae* are termed blastospores. Other asexual spore types include: conidia (nonmotile, typically produced at the tip of a hypha, Fig. 39.3(a), with some fungi producing more than one type, Fig. 39.3(b)); sporangiospores (nonmotile, produced within a sac-like structure termed a sporangium, Fig 39.3(c)); zoospores (motile, with one or more flagella, Fig 39.3(d)), arthrospores (nonmotile, produced by fragmentation of a hypha, Fig 39.3(e)) and chlamydospores (nonmotile, thick-walled, derived from a hyphal compartment, Fig 39.3(f)).

In addition, some of the features of spore-bearing structures are often characteristic of a particular group of fungi: for example, members of the genus *Penicillium* produce chains of conidia from aerial hyphae (conidiophores), with a cluster of conidia-producing cells (termed phialides), forming a structure termed a penicillius (Latin = ‘little brush’, Fig. 39.3(a)) whereas members of the genus *Aspergillus* have phialides clustered on a swollen aerial conidiophore, forming a spherical ‘head’ of conidia. Similar diversity is seen for sexual spores, which include zygospores (in zygomycete fungi – a diploid spore created following the fusion of nuclei from two haploid mycelia, Fig. 39.3(g)) ascospores (in ascomycete fungi – typically

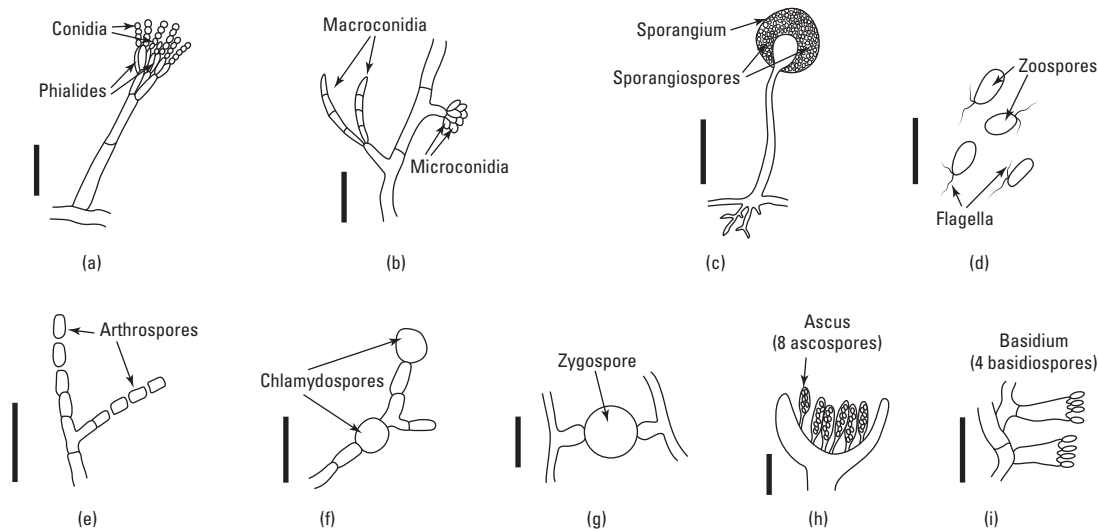


Fig. 39.3 Representative examples of fungal spores: (a) conidia (*Penicillium* sp. Scale bar = 10 μ m); (b) macroconidia and microconidia (*Fusarium* sp. Scale bar = 10 μ m); (c) sporangiospores (*Rhizopus* sp. Scale bar = 100 μ m); (d) zoospores (*Phytophthora* sp. Scale bar = 10 μ m); (e) arthrospores (*Geotrichum* sp. Scale bar = 10 μ m); (f) chlamydospores (*Candida* sp. Scale bar = 10 μ m); (g) zygosporangium (*Mucor* sp. Scale bar = 100 μ m); (h) ascus with ascospores (*Peziza* sp. Scale bar = 100 μ m); (i) basidium with basidiospores (*Agaricus* sp. Scale bar = 10 μ m).

Box 39.1 How to make a fungal slide preparation using lactophenol cotton blue (LCB)

The following procedure covers the main steps involved:

- **Place a small drop of 70% v/v alcohol at the centre of a clean microscope slide.** The alcohol will help avoid the formation of air bubbles in the preparation.
- **Add a small amount of fungal material** – either from a natural sample, or from a pure culture. Yeast cultures can be sampled using an inoculating loop (p. 237) in a similar manner to bacteria. Filamentous fungi require a little more care, due to their more complex, three-dimensional form – using a sterile loop or a pair of dissecting (mounted) needles (p. 237) either tease out a small amount of the mycelium along with the surface of the agar growth medium (approx. 1 mm²). Try to disturb the material as little as possible during transfer to the slide. Your aim should be to sample the edge of the yeast colony or mycelium, as this will include young, growing material.
- **Add a single drop of LCB mountant.**
- **Place a coverslip on your preparation.** Lower from one side (Fig. 41.4) to reduce the risk of air bubbles.
- **View using a light microscope.** Use the low power lens (x10 objective) for initial examination, starting towards the edges of the preparation, where fungal structures will be less likely to be overlaid. Move to higher power

(x40 objective) for detailed examination, once suitable structures have been identified, for example, sporangia/spores.

One variation on the 'tease mount' described above is the slide culture technique, used to better maintain three-dimensional structure of filamentous fungi. Here, small blocks (5 mm × 5mm) are first cut from a thin preparation (1–2 mm deep) of a suitable agar medium, then inoculated with a pure culture of a filamentous fungus and covered with a sterile coverslip. Blocks are then grown under suitable conditions to allow sporulation, then transferred with the coverslip onto a slide containing one or two drops of LCB. Observe the mycelium growing around the edges of the block to locate characteristic shapes of spore-producing structures and arrangement of spores.

Another approach is to use cellophane adhesive tape (e.g. Sellotape®) to make fungal preparations by first gently pressing a small length of adhesive side of the tape onto the surface of a colony (yeast) or mycelium (mould) and then placing this 'fungus-side-down' onto a drop of lactophenol cotton blue on a clean microscope slide. The tape then acts as a coverslip. Alternatively, the mountant can be added to the tape, which is then placed onto a slide 'fungus-side-up', followed by the addition of a coverslip – this avoids the need to view the preparation through a layer of tape.

an ascus encloses eight haploid ascospores, created by meiotic then mitotic division, Fig. 39.3(h)) and basidiospores (in basidiomycete fungi – typically four basidiospores are produced as a result of meiotic division, borne on a cell termed a basidium, Fig. 39.3(i)). While spores can sometimes be seen on the surface of a mycelium using a stereomicroscope, more information can be gleaned by preparing a lactophenol cotton blue mount (Box 39.1) of mature mycelium, and viewing using a standard binocular microscope (p. 176).

Protozoa

These are often viewed as unstained, wet mounts, so that motility can be observed in living cells – the organisms are visible due to the difference in refractive index between cells and medium. An alternative approach is to use the ‘hanging drop’ technique with a drop of suspension on a coverslip placed onto a cavity slide with a concave depression at its centre (the same effect can be achieved with a ring of adhesive putty, such as Blu Tac®). In all instances, you should observe motility near the centre of the preparation. The movement of fast-moving protozoa can be slowed by increasing the viscosity of the mounting fluid, for example using methylcellulose, gelatin or Protoslo®. Other features to look for include means of locomotion (for example, some protozoa move by forming pseudopodia, demonstrating amoeboid motion (Fig. 39.4(a)), whereas others use cilia or flagella (Fig. 39.4(b),(c)) to move) and the operation of contractile vacuoles (organelles that regulate osmotically driving water inflow in protozoa growing in fresh and brackish waters). It is also possible to stain living protozoa; stains used include methylene blue (stains nucleus and cytoplasmic inclusions), toluidine blue (stains cilia/flagella and nucleus) and neutral red (stains nucleus and food vacuoles) – used at low dilution, these stains allow short-term survival, enabling observation of live protozoa. Permanent preparations are made following fixation in glycerinaldehyde or a similar fixative.

Definition

Wet mount – temporary preparation made by suspending the specimen in a small droplet of water or an appropriate liquid, with a coverslip then added (see p. 258).

Microalgae

Because of their photosynthetic pigments, most microalgae can be observed microscopically without staining. Wet mounts can be prepared using environmental samples (for example a plankton net sample of seawater), and lab cultures. Semi-permanent mounts can be prepared using a drop of glycerol – storage time can be extended by sealing the edges of the coverslip using nail varnish. Microalgal cells often have characteristic features, as shown in Fig. 39.5, enabling tentative identification.

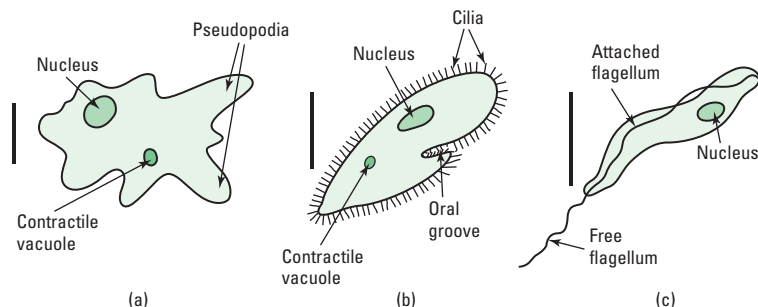


Fig. 39.4 Representative protozoa: (a) *Amoeba* sp. (scale bar = 100 μ m); (b) *Paramecium* sp. (scale bar = 100 μ m); (c) *Trypanosoma* sp. (scale bar = 10 μ m). Contractile vacuoles in *Amoeba* and *Paramecium* will be seen to fill and empty in living specimens.

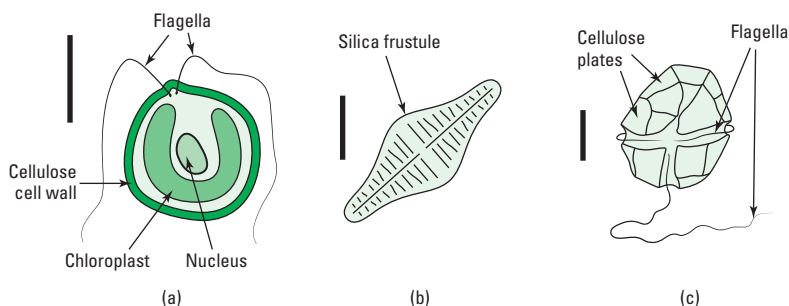


Fig. 39.5 Representative microalgae: (a) *Chlamydomonas* sp.; (b) *Navicula* sp.; (c) *Gonyaulax* sp. (scale bars = 10 μ m).

Culturing eukaryotic microbes

Different groups of eukaryotic microbes have different specific culture requirements. The fundamental aspects are covered below for each group. It is worth noting that the growth rates (doubling times, p. 243) of most eukaryotic microbes are far slower than those of bacteria, meaning that any bacterial contaminants gaining access to cultures are likely to rapidly outgrow the original culture.

KEY POINT Due to the risk of overgrowth of contaminant bacteria, cultures of eukaryotic microbes must be checked regularly for signs of bacterial contamination – for example, by sub-culture onto nutrient agar.

Fungi

Many of the broad principles described for bacterial culture and enumeration in liquid media (broth) and agar-based media (Chapter 38) also apply to unicellular fungi, since yeasts share a unicellular growth form with bacteria, producing colonies on agar-based media and growing as homogeneous cell suspensions in liquid culture.

In contrast, filamentous fungi have a more complex three-dimensional growth form and are mostly grown on the surface of agar-based media, where an initial inoculum will spread radially to form a circular mycelium that extends outwards across the medium, eventually reaching the edge of the Petri plate. The growing tips of the youngest hyphae will be found on and near the surface of the agar at the outermost edge of the mycelium (use to observe actively growing hyphae), while the central region will contain older hyphae and reproductive structures (use to locate spores).

Filamentous fungi can also be grown in broth culture, typically forming either spherical mycelial ‘pellets’ or more dispersed growth of hyphal fragments, depending upon the species and level of agitation/aeration. Such systems are used to produce commercial quantities of biomass, enzymes and antibiotics from filamentous fungi.

Understanding the growth form of moulds – to describe the growing mycelium of a filamentous fungi as a ‘colony’ is a misrepresentation of the term, which should be reserved for microbes such as yeasts and bacteria which grow as visible clumps (colonies), typically derived from a single cell. Refer to such growth as mycelium/mycelia.

The most widely used fungal growth media tend to be slightly acidic, typically pH 6.0 or less, preferentially inhibiting the growth of many bacteria. Sabouraud medium is a typical example, containing:

- **Dextrose** (40 g L^{-1}) – glucose made by hydrolysing corn. Provides the main source of carbon and energy in an easily assimilated form.
- **Peptone** (10 g L^{-1}) – pepsin digest of animal tissue. Source of nitrogen and other elements/compounds.
- **Agar** (optional, at 20 g L^{-1}) – creates a solid medium (Sabouraud agar).
- **Antibiotics** (optional, for example, chloramphenicol/gentamicin/tetracycline) – broad-spectrum antibiotics can be used to inhibit growth of bacteria during primary isolation from environmental samples.
- **pH 5.6.**

Using antibiotics – note that it is neither necessary nor good practice to use antibacterial agents for routine sub-culture of pure isolates/strain of fungi.

Fungi are usually grown aerobically in a temperature-controlled incubator at 20–25 °C for environmental/plant isolates and 37 °C for animal/human pathogens and commensals. Yeasts are typically facultative anaerobes, capable of growth under both aerobic and anaerobic conditions. In contrast, many filamentous fungi are obligate aerobes, growing well on the surface of agar-based media, but requiring agitation and forced aeration ('sparging', p. 242) for optimum growth in broth culture.

Protozoa

Small samples of water, soil, etc. are most often cultured for short periods in the laboratory, under condition that encourage the growth of protozoa – for example by adding sterile natural substances such as hay, wheat or powdered yeast. Examine daily, to follow the dynamics of protozoal growth. You can use the techniques described in Chapter 34, such as a counting chamber for direct microscopic counts (Box 34.1), or an electronic particle counter (pp. 244–6). Pure cultures of protozoa are available from culture collections (for example, Culture Collection of Algae and Protozoa, Cambridge, UK), together with details on appropriate growth media (protozoa are generally regarded as fastidious (p. 270) in pure culture).

Microalgae

These are typically cultured in liquid inorganic media, under an artificial light source – either continuous or on a daily cycle. One example is Guillard's F/2 medium, used for marine microalgae; it contains a defined mixture of specific inorganic salts and vitamins, permitting the growth of a broad range of marine diatoms and other algae.

Perhaps the most important practical aspect of culturing microalgae is the requirement for reduced light levels during initial growth. When setting up a new culture or sub-culture, shade with a layer or two of muslin or baking paper, to prevent photobleaching. Once the culture has started to grow (=visibly greener), you can remove the muslin/paper, as the cells will self-shade, thereby preventing bleaching.

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STUDY EXERCISES

39.1 Devise an experimental protocol to determine effect of temperature on the growth of a filamentous fungus (mould). How would you measure the growth rate of the fungus at different temperatures?

39.2 Calculate the area density of spores on the surface of the gills of a basidiomycete fungus (mushroom). A sample of gill from *Agaricus campestris* was counted using an oil immersion (x100) objective with field diameter of 180 μm , giving an average count of 279

spores per field of view. Determine the number of spores per mm^2 .

39.3 Determine the speed of travel of a motile protist. A dinoflagellate was observed to travel halfway across a microscope field of view of diameter 825 μm in 11 s. Calculate its speed in $\mu\text{m s}^{-1}$. How does this compare with human walking speed (approximately 4 miles (6.5 km) per hour)? Refer to Box 26.1 for interconversions between units.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

40 Working with viruses

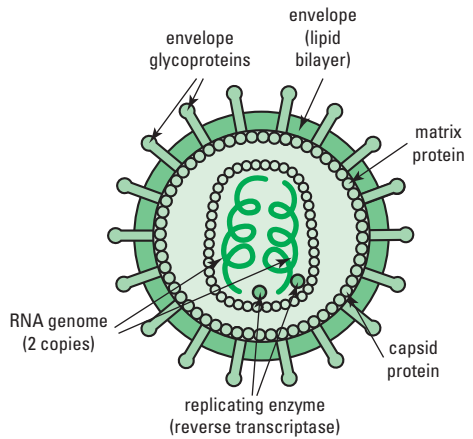


Fig. 40.1 Structure of the HIV virion, showing major components. Scale bar = 100 nm.

Viruses are sub-cellular particles consisting of a core of nucleic acid (genome) surrounded by a protective protein coat (capsid) – together these comprise the nucleocapsid. Some animal viruses have a further membranous layer, termed the envelope (Fig. 40.1), while others contain copies of enzymes involved in replication. Each virus particle is termed a virion. Without their own metabolic systems, viruses are obligate intracellular parasites; typically, one virion infects a single susceptible host cell, using the cell's metabolism to transcribe and translate genes within the viral genome to generate hundreds of new virions in a process termed replication. The process is also known as a one-step growth cycle.

Since an individual virion is typically 20–500 nm in linear dimensions, it cannot be seen using a light microscope and high-power objective. Virions can only be visualised using an electron microscope (p. 172). Consequently, practical procedures often involve *indirect* observation of the consequences of viral infection for host cells, such as cell degeneration and death (cytopathic effects), or the detection and quantification of relevant biomolecules, such as viral nucleic acids or capsid proteins.

Taking account of the diversity of viruses

Viruses can be sub-divided in several different ways, including by:

- **Host cell type.** Different viruses infect every group of cellular organisms, whether eukaryotes or prokaryotes: animal viruses are different to plant viruses, which in turn differ from those infecting fungi (mycoviruses). Bacteria and Archaea have their own specific types of viruses. Bacterial viruses are often termed bacteriophages, or simply 'phages' – so named because they destroy ('eat') bacterial cells (Greek: *phagos* = eater). Within all of these groups, viruses vary in their host specificity, determined by the interaction between surface proteins on the virion and receptors on the surface of target host cells. Some viruses have a narrow range (for example, human immunodeficiency virus, HIV, which infects a subset of cells of the human immune system that have CD4 receptors on their surface), whereas others have a broader range (for example, the causative agent of COVID-19, SARS-CoV-2, infects cells through the ACE2 receptor, which is found on cells of domestic and wild animals, in addition to human cells). Note also that some viruses can become latent within infected cells, often by incorporation of the virus genome into the host genome as a provirus: such dormant viruses can then reactivate at a later stage, causing lysis of infected cells. Human examples include herpes simplex virus (HSV) and Epstein–Barr virus (EBV). Some viruses can also cause *transformation* of the host cell, typically causing indefinite growth of the transformed cell, as in virally mediated human cancers (for example, genital cancers due to human papilloma virus, HPV). Host range is an important aspect of practical virology, with great care required when handling human and animal viruses in the laboratory.
- **Disease symptoms.** Often the disease is used as part of the name of a new virus; examples include human severe acute respiratory syndrome coronavirus (SARS-CoV) and potato virus X (PVX). Further details of naming and classifying viruses are given in Chapter 37 (p. 267).

Recognising popular misconceptions

of human viruses – depictions of tailed viruses entering human cells often seen in popular media are incorrect, as this form is only found among bacterial viruses. Human viruses typically enter cells either by fusion of the envelope with the host plasma membrane, or via endocytosis for non-enveloped virions.

Disease symptoms are an important characteristic in identifying pathogenic viruses.

- Morphology.** Viruses can be described as: filamentous (Fig. 40.2(a)), where the capsid protein subunits are arranged in a spiral around the nucleic acid core; icosahedral (Fig. 40.2(b)), where the capsid proteins form a twenty-sided icosahedron; enveloped (complex animal viruses, Fig. 40.1), where the nucleocapsid is surrounded by a phospholipid bilayer derived from host cell membrane, plus virus proteins/glycoproteins; and tailed (complex phages, Fig. 40.2(c)), where an icosahedral nucleocapsid ‘head’ is attached to a contractile ‘tail’ that mediates attachment and subsequent penetration of the bacterial cell wall. Note that tailed virus particles only infect prokaryotes (archaea or bacteria). The morphologies of different virus groups are often used within their names; for example, rotaviruses (Latin ‘*rota*’ = wheel) have a wheel-like shape, when viewed with an electron microscope, while coronaviruses (Latin ‘*corona*’ = wreath/crown) have a fringe of surface projections (spike proteins), giving them their characteristic appearance as shown in Fig. 40.3(a)).
- Genome.** There is considerable diversity in organisation of the genetic information within the virion of different groups of viruses. Genomes can be: either DNA or RNA (never both); double-stranded or single-stranded; linear or circular; segmented (several pieces of nucleic acid) or non-segmented (a single molecule of nucleic acid). The Baltimore system of virus classification sub-divides viruses into seven groups, based on nucleic acid type and means of replication (for further details, see Bruslind, 2020). Genomic organisation represents the most fundamental type of information for viruses; consequently nucleic acid sequence data are used increasingly as the primary basis of classification and taxonomy.

KEY POINT You are most likely to carry out practical work using viruses that do not cause human disease, for example, using bacteriophages or plant viruses to minimise your risk of infection.

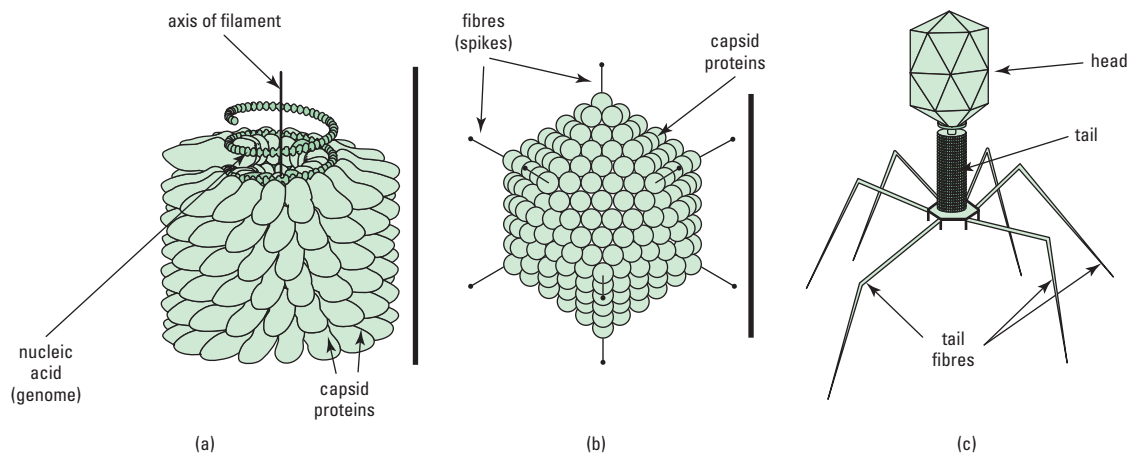


Fig. 40.2 Three major morphological types of virus: (a) filamentous (Tobacco mosaic virus); (b) icosahedral (Adenovirus); (c) complex bacteriophage (T_2 phage of *E. coli*). Scale bars = 100 nm.

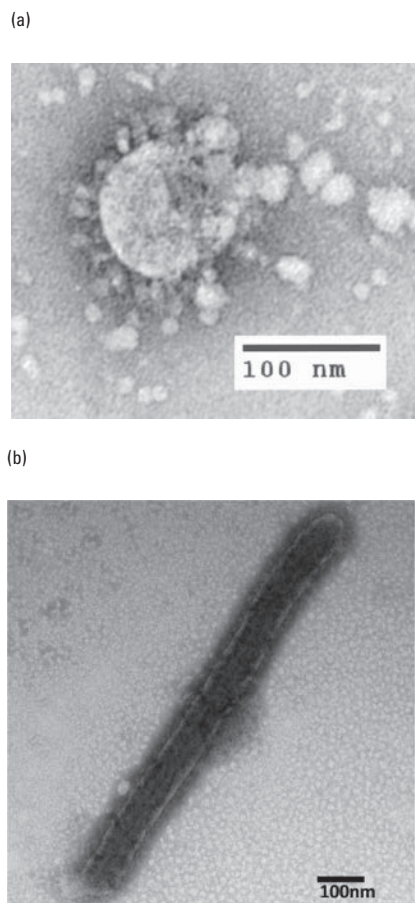


Fig. 40.3 Electron micrographs (negative staining) of (a) SARS-COV-2 and (b) Ebola virus.

Example For a sample with a count of latex particles of 8 per field of view, a virus count of 24 per field of view and a volume density of latex particles of 2000 per μL , eqn [40.1] gives a virus volume density of: $(2000 \times 24)/8 = 6000$ virions per μL .

Using electron microscopy to study viruses

Transmission electron microscopy (TEM, p. 170) is a widely used method for the detection of viruses, avoiding the requirement for culture of infected host cells, and giving a faster result than cell culture. TEM provides useful information on virus morphology that has several applications, including: as an aid to diagnosis for cases of disease; as a measure of contamination for laboratory cell cultures; and as a research tool, for example, when investigating virus entry, assembly and exit from host cells. As TEM requires specialised equipment and expertise, you are most likely to encounter it as a demonstration of the technique, or through electron micrographs of negatively stained preparations of different types of virus (for example, Fig. 40.3). TEM can also be used to provide counts of virus particles per unit volume, typically by mixing an equal volume of virus suspension with latex or gold beads of known size and number per unit volume – TEM counts of latex/gold particles and virus particles across the same fields of view (=unit area) can then provide a measure of virus volume density, using the relationship:

$$\text{Virus particles per unit volume} = \frac{\text{Latex particles per unit volume} \times \text{Virus count per unit area (TEM)}}{\text{Latex particle count per unit area (TEM)}} \quad [40.1]$$

One disadvantage of counts of viruses based on TEM is that they are often higher than methods based on cell culture, for a number of reasons, including:

- **Virus surface proteins may be damaged** and thereby unable to attach to or infect host cells.
- **Virus genomes may be defective.** Errors may occur during the production of new copies of the genome, including mutations and defective replication – the latter can lead to virions with non-functional genes. Mutations can reach high proportions in those viruses with poor ‘proof-reading’ during replication. Detecting such changes in genome sequence can also help track the progress of new variants during epidemiological investigations (pp. 289–91), as for example, during the COVID-19 pandemic.
- **Empty capsids and/or envelopes may be produced.** These may lack any nucleic acid, or may incorporate random fragments of host cell DNA or RNA. In all such cases, they will be unable to replicate in a new host cell.
- **Cell defence systems may neutralise some virus particles.** This will lead to a lower count from cell culture than from TEM, since the former will only result in a count for virions that successfully enter and replicate within a host cell.

Isolating and purifying viruses

It is often necessary to purify virus particles, so that they can be further characterised. Virions can be separated from host cell components by precipitation – for example, using high M_r polyethylene glycol such as PEG-6000. An alternative approach is to use ultracentrifugation (p. 320), with differential sedimentation (p. 320) as the first stage, to remove larger debris and provide a pellet that is enriched in virus particles followed by further stages, typically density-gradient ultracentrifugation (pp. 320–1), using either rate-zonal ultracentrifugation with a stepped gradient of 10–40%

sucrose, or isopycnic procedures using a 30–55% CsCl gradient. TEM can be used to assess purity at different stages of the process.

Gel permeation chromatography (p. 352) can be used to purify virions or their constituent subunits, including surface proteins (antigens) and enzymes. Sepharose (agarose) columns are used as a second step, following initial ultracentrifugation. Elution of virus particles can be followed by measuring absorbance at 280 nm, with confirmation by TEM or using a nucleic acid probe.

Detecting viruses using immunological tests

A wide variety of different methods can be used, depending on the purpose of the investigation. These include:

Definitions

Serum – the fluid remaining when blood has been allowed to coagulate. Serum is more stable than blood and contains soluble antibodies that can be detected using serological testing.

Titre – a measure of either the amount or concentration of a substance in a solution (from the same origin as 'titration'). In virology, titre typically refers to the highest dilution of an immunological test showing a positive result, or to plaques/foci of infection in a cell culture.

- **Virus neutralization.** This is a serological test, used to detect the presence of virus-neutralising antibodies in serum samples, produced in response to the presence of viral antigens. A virus suspension can be mixed with the test serum and left to react for up to 1 h. If the test serum contains antibodies that block virus replication (neutralisation), the mixture will not infect cells of the host organism. Infectivity is typically studied using animal or human cell cultures.
- **Latex agglutination.** This detects the presence of a particular virus in a test sample – commercially produced latex microbeads coated with a virus-specific antibody (p. 393) are mixed with the test sample, with a positive reaction detected as a visible clumping of the microbeads.
- **Haemagglutination.** This is used for those animal/human viruses with surface proteins (haemagglutinins, HA) that cause clumping of red blood cells, for example, influenza virus. The standard (direct) haemagglutination test detects the presence of a virus in a test sample: serial doubling dilutions (p. 147) of the sample are mixed with red blood cells in a microtitre plate and left to react – when a virus is present in sufficient quantity, the red blood cells agglutinate, settling in a diffuse layer, while at greater dilution of the virus, they coagulate to form a 'button' of coagulated cells. This enables the 'titre' (the last dilution to show haemagglutination) to be determined. In the haemagglutinin *inhibition* test, haemagglutinin-neutralising antiviral antibodies are detected in test serum.
- **Immunoassays.** These include enzyme immunoassays (EIA, p. 396) and enzyme-linked immunoassays (ELISA, p. 396) and dipstick immunoassays (pp. 397–9) for the detection of either viral antigens or antiviral antibodies, depending upon the particular format of the test. EIA and ELISA are more sensitive than most other immunological tests and can provide quantitative information on amount of antigen or antibody present in the test sample.
- **Fluorescent antibody staining.** This can be used to detect the presence of virus antigens within infected host cells. Cells under test are first 'fixed' using an organic solvent, making the cell membrane permeable to a monoclonal antibody (p. 393) labelled with a fluorophore such as fluorescein isothiocyanate (FITC). The presence of viral antigens within infected cells is visible by their fluorescence under UV microscopy.
- **Protein immunoblotting** – sometimes termed Western blotting (cf. Southern blotting, p. 372), antibodies are reacted with viral proteins on

filter membranes, for example after electrophoretic protein separation (Chapter 50), with detection using enzyme-labelled or fluorogen-labelled antibodies.

- **Complement fixation.** This can be used to detect virus antigens or antiviral antibodies. The two main advantages are (i) increased sensitivity, compared to standard antigen/antibody tests and (ii) the capacity to screen for several different viruses at the same time. For more detail, see Chapter 53.

Studying viral nucleic acids

The methods used follow the broad procedures outlined in Chapter 67. Purification typically includes:

- **Pre-filtration** – using a 0.2 µm filter to remove cells and cell debris, and thereby reduce the amount of non-viral nucleic acid. However, this step can also remove some viral nucleic acid. Centrifugation is an alternative approach.
- **Enzymic digestion** – viral envelopes/capsids can be disrupted using proteinases, often in the presence of nuclease inhibitors to prevent degradation of viral genomes.
- **Adsorption-elution** – typically, using anion exchange material or silica. As an example, the silica-based ‘spin minicolumns’ produced by QIAgen (QIAamp®) and Promega (Wizard®) use this approach, avoiding the need for traditional preparation of nucleic acids using phenol-chloroform extraction and alcohol precipitation by carrying out binding, washing and elution of nucleic acids within a 1.5 mL microcentrifuge tube (p. 321), using microcentrifugation to drive each step of the process.

Once purified viral nucleic acid has been obtained, it can be characterised further, for example sizing using polyacrylamide gel electrophoresis (PAGE, Chapter 50), or Southern/Northern blotting to detect particular sequences of DNA/RNA (Chapter 67).

PCR-based amplification and detection of viral nucleic acid sequences has the advantage of speed of assay when compared to cell culture, achieving an outcome within hours rather than days. The primers used target unique sequences for a particular virus, with detection of amplified sequences by PAGE or dot blotting (p. 517). Nested PCR, using two sets of primers and two PCR stages, can be used with the aim of increasing specificity and sensitivity (for further detail, see Carr *et al.*, 2010). Reverse transcriptase-PCT (RT-PCR) can be used with RNA viruses. Quantitative PCR (qPCR) uses fluorescent dyes/probes and a fluorometer to monitor the amplification process in real time, hence its alternative name of ‘real time’ PCR – for further details, see Chapter 68.

Studying viruses using cell culture: phages

A major advantage of methods based on cell culture is that they detect only infective virus particles. Bacterial viruses (phages) are often used to illustrate the general principles involved in the detection and enumeration of viruses by cell culture. Phages are safe to handle in practical classes, typically used with bacteria in ACDP Hazard Group 1 (p. 238), for example non-pathogenic strains of *Escherichia coli*. They are sub-divided into two groups, according to their effects on susceptible host cells:

Tracking mutations in virus genomes – nucleic acid sequencing (p. 285) can be used to detect mutational changes through time. As an example, SARS-CoV-2 exhibits error-prone replication, resulting in mutations across all of the major viral proteins. These mutations have been studied in epidemiological investigations of the origins, distribution, pathogenicity and dissemination of different variants (mutants), as part of the response to the 2019 COVID-19 pandemic.

Culturing animal and human viruses – in addition to primary cell cultures and continuous cell lines (Chapter 41), whole animals and embryonated hen’s eggs are used to cultivate some viruses.

Definitions (epidemiological terms)

Asymptomatic – infected, but currently showing no symptoms. Can also refer to different stages of infection, including pre-symptomatic (early stages) or post-symptomatic (carrier).

Epidemic – an increase in cases of a disease within a defined population of a specific geographical area.

Herd immunity – resistance of a population to a disease resulting from a significant number of individuals being immune (resistant to infection, often due to post-infection immune response).

Index case – the first case identified in a specified population. Sometimes referred to as ‘patient zero’.

Incidence – the number of new cases of a disease that develop in a population within a specified time, typically a day, month or year.

Pandemic – an epidemic involving the worldwide spread of a disease, typically due to the emergence of a new form of a pathogen.

Prevalence – all of the cases of disease within a population at a specific time point, sometimes expressed as a percentage.

Reproductive number (R) – a parameter describing the contagiousness (transmissibility) of a disease. The average number of individuals infected from a single case.

Shedding – release of new infective agents (e.g. virions) from the host (e.g. from respiratory tract, alimentary tract, skin)

Symptomatic – showing symptoms of a disease as a result of infection.

Vaccine (from Latin *vacca* = cow, since cowpox was used as the first vaccine against smallpox virus) – a substance used to stimulate the production of antibodies and hence prevent or ameliorate a disease through the development of host immunity. Typically, developed from weakened or inactivated forms of the pathogen, or from surface components.

Vector – an organism that transmits a disease from one organism to another.

- **Virulent phages** – will infect and replicate within actively growing host cells, causing cell lysis and releasing new infective phages – this ‘lytic cycle’ takes ≈ 30 min for phages of *E. coli*, such as T4 phage, a widely used example of a tailed lytic phage.
- **Temperate phages** – a specialised group, capable of either (i) lytic growth or (ii) an alternative process, termed lysogeny – the phage becomes latent within a host cell (which is termed a lysogen, since it is capable of *generating lysis*). A lysogenic phage typically shows incorporation of its genetic information into the host cell genome, becoming a ‘prophage’. At a later stage, termed induction, the prophage may enter the lytic cycle. A widely used example is λ phage of *E. coli*.

Box 40.1 gives details of a plaque assay, used to determine the number of phages present in a test sample.

Studying viral epidemiology

As viruses are obligate pathogens, their influence on the transmission of disease within their hosts can be studied using epidemiological methods. The text below covers the basic principles, using human viruses as example.

Table 40.1 lists the major viral pandemics that have occurred since 1900. For many of these pandemics, the emergence of novel diseases has been associated with animal-to-human transmission, with new strains of animal viruses being the major source of novel and emerging pathogens of humans. However, not all epidemics are caused by viruses. Two recent examples of recent epidemic bacterial diseases are ‘Legionnaires’ disease (*Legionella pneumophila*) and methicillin-resistant *Staphylococcus aureus* (MRSA).

Understanding the epidemiology of infectious disease

This branch of science deals with the distribution, dynamics and determinants of disease in a specified population. Typically, it involves the investigation of the factors determining adverse health events (disease outbreaks) in populations and the application of this understanding to help control or prevent them in future. Epidemiology is closely connected with virology, since many pandemics have involved viruses as agents of infection. It involves data analysis and the use of mathematical modelling and

Table 40.1 The major viral pandemics of the 20th and 21st centuries. The agent of disease is noted in brackets. The date given is the approximate start of the pandemics and not necessarily the first case

Date	Epidemic
1918	Influenza – ‘Spanish Flu’ (H1N1 virus)
1957	Influenza – ‘Asian Flu’ (H2N2 virus)
1968	Influenza – ‘Hong Kong Flu’ (H3N2 virus)
1979	Human immunodeficiency virus – acquired immune deficiency syndrome – HIV/AIDS (Lentivirus HIV-1 and HIV-2)
2002	Severe Acute Respiratory Syndrome – SARS (SARS-CoV-1 virus)
2012	Middle East Respiratory Syndrome – MERS, sometimes termed ‘camel flu’ (MERS-CoV virus)
2013	Ebola (West Africa) – Ebola virus disease or EVD (Ebola virus)
2019	Coronavirus – ‘COVID-19’ (SARS-CoV-2)

Box 40.1 How to carry out a plaque assay using a bacteriophage

You will typically be given a test suspension of phages to enumerate, plus a susceptible host cell. Typically, a lytic phage such as T4 is used to demonstrate the procedure in practical classes using *E. coli* as host, as follows:

- **Mix a known volume of sample with a small volume of susceptible bacterial cells in molten soft agar medium** (45–50 °C). The concentration of agar used is typically half the strength of 'normal' agar, to allow phages to diffuse through the medium and attach to susceptible host cells.
- **Pour the mixture onto a plate of the same medium, creating a thin layer of 'top agar'.**
- **Incubate the plate** (for example, at 37 °C overnight for *E. coli*). The bacteria will grow throughout the top agar layer to produce a homogeneous 'lawn' of cells, except where a phage particle has infected and lysed the cells to create a clear area, termed a plaque (Fig. 40.4).
- **Count the number of plaques.** View plates against a black background to make plaques easier to see: mark each plaque with a spirit-based marker as you count, to ensure accuracy. Lytic phages will give clear plaques, while temperate phages often produce cloudy plaques, because many of the infected cells will be lysogenised rather than lysed, creating turbidity within the plaque. Each plaque is due to a single functional phage (i.e. a plaque-forming unit, or PFU). The plaque count can be used to give the number of phages in a particular sample (e.g. as PFU mL⁻¹), with appropriate correction for dilution and the volume of sample counted in an analogous manner to a bacterial plate count (p. 246). The relationship is:

$$\text{PFU mL}^{-1} = \text{mean plaque count per plate} / \text{volume of sample added to plate} \quad [40.2]$$

For example, for an undiluted sample with a mean plaque count per plate of 42 for a volume of 0.05 mL of sample, the count would be:

$$42 / 0.05 = 840 \text{ PFU mL}^{-1}$$

Correct for any dilution by multiplying by the reciprocal of the dilution factor – for example, if the above calculation had been for a 10⁻² dilution, the original sample would have had a count of $840 \times 10^2 = 84000 = 8.4 \times 10^4 \text{ PFU mL}^{-1}$.

- **Where required, harvest progeny virions from a single plaque** using a Pasteur pipette (p. 143) or an inoculating needle, providing a clone from a single original virion. This can be used to subculture the phage for further study, for example, in a broth culture where the phages will cause widespread cell lysis and a decrease in turbidity. Alternatively, phages can be stored by adding chloroform to aqueous suspensions – this will prevent contamination by cellular microorganisms.

A broadly similar approach can be used to detect and count animal or human viruses, using a monolayer of susceptible host cells – plaque visualisation usually requires staining with a suitable dye, with plaques (foci of infection) showing as unstained areas against a background ('lawn') of uninfected, stained cells.

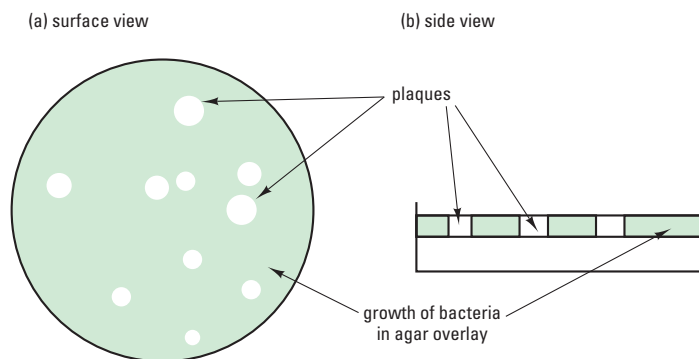


Fig. 40.4 Phage plaques in a 'lawn' of susceptible host bacterium.

statistics, combined with broader approaches from social and behavioural sciences. Box 40.2 provides an example of how a disease outbreak can be simulated in a practical class.

Epidemiologists seek to understand the frequency and pattern of disease events, which essentially involves finding answers to the following questions:

1. What is the nature of the disease?
2. Who does it involve?
3. Where does it occur?
4. What is the time-course?
5. What are the causes of the disease?
6. What risk factors are involved?
7. What are the modes of transmission of the disease?

Each of these questions can only be answered by collection and analysis of relevant data.

Mathematical modelling in epidemiology

This is used to describe the spread of diseases and to assist in assessing the effectiveness of strategies for controlling an epidemic. A key parameter in many models is R the reproductive number. This describes the average number of people an infected individual will infect in a totally susceptible population. If R is greater than one, then the outbreak will expand, if equal to one, then it will be static, and if less than one it will decline. R can be calculated as:

$$R = \beta\tau \quad [40.3]$$

where where β = infection-producing contacts per unit time and τ = mean infectious period. Eqn. [40.3] also points towards approaches that can reduce R for infectious diseases, for example, quarantine of infected individuals will decrease β , while treatment of the infection will decrease τ . Other models for the spread of infections exist, but they involve complex mathematics, and a specialist text should be consulted (for example, Walters *et al.*, 2018).

KEY POINT It is important to recognise that any mathematical model for describing an epidemic is only as good as the assumptions behind it *and* the estimated values of the parameters entered in the model.

Box 40.2 provides an example of how a disease outbreak can be simulated in a practical class.

Box 40.2 How to model disease transmission in a practical class

An outbreak of disease can be simulated in a lab class by a variety of approaches: the example below uses T4 phage as the simulated pathogen and *E. coli* as the detection system (host) and is, therefore, easily tracked in the laboratory. Alternative approaches include using bacteria that give a characteristic colour on a particular agar medium (simulated bacterial outbreak), different coloured beads, (generic model of disease outbreak). The version described below involves pipetting of fluids between test tubes to simulate transfer of body fluids, such as might happen when a virus is released from a mucosal surface of one individual to the mucosal surface of a second individual, sometimes involving objects such as clothes, furniture, household surfaces (these are sometimes termed 'fomites'). The basic procedure using T4/*E.coli* is:

- 1. You will be given a test tube containing a clear liquid (your simulated body fluid), labelled 'SBF'** – one of the SBF tubes given to students in the class will have been inoculated with a suspension of T4 phage (=index case). The remaining tubes of SBF will be uncontaminated at the outset (=healthy individuals). Add your initials to your tube of SBF. You will use this to exchange body fluids (=SBF) with others in the class.
- 2. Using a sterile Pasteur pipette, transfer a single drop of your SBF to a tube of sterile nutrient broth. Label this tube with your initials and 'round 0'.**
- 3. 'Partner up' with another student and exchange SBF by transferring six drops of your SBF to your partner's SBF tube, then six drops of their SBF to your SBF** (it doesn't matter who goes first). If one of you has SBF containing T4 phage, this will then 'infect' the SBF of the other partner through the exchange of SBF.
- 4. Transfer a single drop of your SBF to a fresh tube of sterile nutrient broth. Label this tube with your initials and 'round 1'.**
- 5. Repeat steps 3 and 4, selecting a different student for each new 'round'. Label each new tube of nutrient broth to show the round number.**
- 6. At the end of the process (either a fixed number of rounds, or once all students have exchanged SBF), add**

a few drops of an overnight broth culture of *E. coli* to every tube of nutrient broth. The *E. coli* will act as an indicator of disease (=presence of T4 phage), showing clearing of the broth in the presence of T4 phage, while broth without T4 will be turbid, due to growth of healthy *E. coli*.

- 7. Incubate at 37 °C overnight, then score each tube of nutrient broth as either clear (= disease case) or cloudy (= healthy individual).**
- 8. Collect the class data for all rounds.** Examining the results for the 'round 0' set of tubes will identify the index case. Subsequent rounds can be considered as a time series, showing how the disease spread across the group; this can also be plotted as a graph of the outbreak over time.

The experiment described above can be modified in several different ways, including:

- Dividing the class into small groups, rather than as a whole-of-class exercise (reaching '100% infected' in a reduced number of rounds).
- Having more than one case in 'round 0' (faster increase in case numbers in subsequent rounds).
- Having students exchange SBF with more than one other student per round (simulating outbreaks where $R > 1$).
- Having some students who are immune to infection; for example, a 'vaccine' comprising a different bacterium (e.g. *Lactobacillus*) that is not susceptible to T4 can be given to some SBF tubes, whereas others are given a placebo of killed *Lactobacillus*. Those given the vaccine (live *Lactobacillus*) will produce cloudy broths throughout, irrespective of whether they also contain T4, whereas those given the placebo will still show clearing in the presence of T4.

Note that it is important to follow sterile technique (p. 235) to avoid any growth of contaminants, which would result in cloudy tubes independent of the effects of T4 phage on *E. coli*, thereby invalidating the results.

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STUDY EXERCISES

40.1 Calculate the volume density of a suspension of virions based on microscopic counts. Using eqn [40.1], determine the number of virions per mL of a sample that was mixed in equal quantities with a latex particle suspension of 2500 particles per μL , giving microscopic counts of 29 latex particles and 71 virions per field of view.

40.2 Calculate plaque-forming units (PFU) per mL of original sample, based on plaque counts. Using eqn [40.2], determine PFU mL^{-1} for triplicate 50 μL aliquots

of a fifty-fold dilution of a virus suspension that gave counts of 56, 63 and 61 plaques per plate.

40.3 Interpret R values for different scenarios. Two different locations had a similar daily count of 100 new COVID-19 cases per thousand head of population, with R values of 0.8 (location A) and 1.6 (location B). How would you expect the daily case numbers to change in each location in subsequent days?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

41 Working with animal and plant tissues and cells

Definitions

In vitro – occurring outside a living organism, in an artificial environment (literally ‘in glass’).

In vivo – occurring within a living organism (literally ‘in life’).

Examples Areas where cell culture methods are valuable:

- **Toxicity tests**, where the use of mammalian cell culture avoids the ethics and costs associated with whole animal experimentation (Chapter 30).
- **Vaccine production**, where, for example, mammalian cell lines have been used in the development of SARS-CoV-2 vaccines (Chapter 40).
- **Molecular genetics**, where animal and plant cells and protoplasts can be used in genetic manipulation (Chapter 69).

Working with vertebrate animals and their organs/tissues – remember that procedures must be consistent with the law, i.e. in the UK, the *Animals Scientific Procedures Act 2013*. This may be referred to by its acronym ASPA.

Using human tissues – in the UK, the *Human Tissue Act 2004* or HTA (revised in 2019 and 2020) applies and work is licensed and overseen by the Human Tissue Authority. Note that established cell lines are excluded from the regulations.

Table 41.1 Composition of Ringer’s solution (simplified formulation, for amphibians, etc., pH 7.4–7.6)

Compound	Concentration (g L ⁻¹)
NaCl	6.0
KCl	0.075
CaCl ₂	0.1
NaHCO ₃	0.1

While you can learn much by isolating and studying individual molecules from biological systems (Chapter 56), this is not always the most relevant course of action. You may find it more appropriate to study the functioning of biomolecules within more complex systems, to better understand their role in a particular biological process. At one extreme, you can do this *in vivo*, using whole multi-cellular organisms (for example, individual animals or plants), while the other extreme is represented by *in vitro* studies, using subcellular (cell-free) extracts. Between these two extremes, tissue and cell culture techniques offer some of the biological complexity of the intact organism combined with a degree of experimental control that may not be obtainable *in vivo*.

Working with animal tissues and organs

Physiological experiments are carried out using either whole organisms, or a range of animal organs and tissues, including heart, liver, muscle, etc.

KEY POINT A major practical consideration is that the tissue should be studied as soon as possible after the death of the animal, typically under laboratory conditions that mimic the *in vivo* environment as closely as possible.

In most instances, the experiments are relatively short term (<24 h) and the aim is to maintain the tissue in a physiological state similar to that within the living organism. For metabolic studies, the whole organ or a tissue slice (typically 1–10 mm thick) will be bathed in an appropriate perfusion fluid, supplied either by gravity or by peristaltic pump. Practical aspects you should consider include:

- **Inorganic solute requirements** – the chemical composition of the perfusion fluid is usually chosen to reflect the major inorganic ion requirements of the tissue. For short-term studies, a number of so-called ‘physiological salt solutions’ may be used, for example, Ringer’s solution, one formulation of which is given in Table 41.1.
- **Oxygen requirements** – it may be necessary to increase the O₂ content of the perfusion fluid by bubbling with air, in order to meet the oxygen demand of the innermost parts of the tissue. However, this can lead to oxygen toxicity in the outermost parts and an alternative approach is simply to increase the rate of perfusion.
- **Physico-chemical conditions** – including temperature (usually controlled to $\pm 1^\circ\text{C}$ of normal body temperature), water status (the perfusion fluid and the tissue should be isotonic, p. 159), pH and buffering capacity (for example, some perfusion fluids have an elevated NaHCO₃ concentration, to mimic the buffering capacity of mammalian serum).
- **Organic nutrient requirements** – for longer-term studies, suitable nutrients will be required: these may be chemically defined additives, for example, vitamins, amino acids, proteins, etc., or biological fluids such as plasma or serum. Glucose is often added as a carbon and energy source (Freshney, 2015).

Definitions

Apoplast – that part of the plant body outside the symplast.

Light compensation point – the amount of photosynthetically active radiation (PAR) where photosynthetic CO₂ uptake is balanced by CO₂ production due to respiration and photorespiration.

Plasmodesmata – transverse connections through the cell wall, linking the cytoplasm of adjacent plant cells and creating a symplast.

Working with plant tissues and organs – the ethical problems associated with animal and human tissues are avoided, and there is a decreased risk of infection to the laboratory worker; practical manipulations are often carried out in horizontal laminar-flow cabinets (p. 238).

Table 41.2 Components of Long Ashton medium (nitrate version)

Stock solution: mass of component required per litre of solution (g)	Volume of stock solution to make 1 L of medium (mL)
Major nutrients	
KNO ₃ : 50.60	8
Ca(NO ₃) ₂ : 80.25	8
MgSO ₄ ·7H ₂ O: 46.00	8
NaH ₂ PO ₄ ·2H ₂ O: 52.00	4
Micronutrients	
FeKEDTA: 3.30	5
MnSO ₄ ·4H ₂ O: 2.23	1
ZnSO ₄ ·7H ₂ O: 0.29	1
CuSO ₄ ·5H ₂ O: 0.25	1
H ₃ BO ₃ : 3.10	1
Na ₂ MoO ₄ ·2H ₂ O: 0.12	1
NaCl: 5.85	1
CoSO ₄ ·7H ₂ O: 0.056	1

Taking account of the effects of human presence on plants – remember that your exhaled breath will be nearly saturated with water vapour and will contain CO₂ at 3–4% v/v, some 100 times more concentrated than atmospheric CO₂; your breath can thus affect rates of transpiration and photosynthesis.

Working with plant tissues and organs

Individual plant components (for example, leaves, leaf slices and epidermal strips) can be isolated from the main plant body for study under controlled conditions. Since photosynthetic plant parts are autotrophic, they may be maintained *in vitro* for longer than animal organs, given adequate light and CO₂. However, most plant cells are joined via plasmodesmata, and the separation of such connections when the component is removed from the plant often leads to death when these connections are broken.

KEY POINT Plants show wound responses that may affect the metabolic processes under study. For these reasons, the most suitable systems for longer-term studies are often whole organs, e.g. whole leaves or entire root systems.

Water culture (hydroponics), for example, in Long Ashton medium (Table 41.2), is an alternative approach, offering greater control over the root environment (Park, 2021).

You should use vigorous, healthy stock plants and to follow a well-established procedure, taking account of the following:

- **Sterility** – strict attention to sterile technique can be essential to the success of many long-term experiments. Decontamination of plant organs may be especially difficult where specimens are obtained from soil: to achieve this, use a surface wash with disinfectant (for example, 10% w/v sodium hypochlorite), followed by several rinses with sterile water.
- **Gaseous environment** – in general, the experimental system should be well ventilated. Actively photosynthetic tissues will rapidly deplete the atmospheric CO₂ in a closed vessel: plant tissues may also produce physiologically active gases, such as ethylene, especially at wound sites. Turgor loss may occur in isolated plant cells unless a high humidity is maintained.
- **Nutrition** – plant tissues may benefit from a supply of inorganic ions, including K⁺, SO₄²⁻, etc., and may require certain vitamins, micronutrients and plant hormones for prolonged studies.
- **Physico-chemical conditions** – light is the most important environmental requirement for green plant parts. At atmospheric CO₂ concentrations, the light compensation point (p. 295) is about 5–10 μmol photons PAR m⁻² s⁻¹ and photosynthesis is usually saturated between 500 and 2000 μmol photons PAR m⁻² s⁻¹, depending on the plant type (Chapter 64). Light quality and photoperiod (daylength) are also important: LED, fluorescent, and incandescent lights that mimic the photosynthetic spectrum of sunlight are available (see p. 326), while you can control photoperiod using a timer. You can adjust the water potential of aqueous media using an impermeant osmoticum such as mannitol, and maintain pH values close to those of the apoplast (≈ pH 6) using appropriate buffers, if necessary.

Using cell and tissue cultures

Many of the basic principles involved in culturing animal and plant cells are broadly similar to those described for microbial cell culture.

Definitions

Cell line – a cell culture derived by passage of a primary culture.

Clone – a population of cells derived from a single original cell, i.e. sharing the same genotype.

Confluence/confluent growth – merging of individual cells to form a continuous layer.

Continuous cell line – a culture with the capacity for unlimited multiplication *in vitro*. Sometimes termed an established cell line.

Finite cell line – a culture with a limited capacity for growth *in vitro* (maximum number of cell doublings).

Hybridoma – a hybrid cell produced by fusion of a tumour (myeloma) cell and an antibody-producing B lymphocyte (p. 82). A cell line derived from a single hybridoma will produce a single antibody type (monoclonal).

Immortalisation – conversion of a finite cell line into a continuous cell line.

Passage – an alternative term for subculture.

Primary culture – a cell culture derived from tissue or organ fragments (explants). Primary culture ends on first subculture.

Senescence – the end point in the limited lifespan of a finite cell line, characterised by the lack of proliferation. Conversion to an established (continuous) cell line requires 'escape' from senescence.

Stem cell – an undifferentiated animal cell that can generate new stem cells through mitosis, or can develop and differentiate into a particular specialised cell type.

Transformation – a permanent alteration in the growth characteristics of a finite cell line that may include (i) changes in morphology, (ii) an increased growth rate and/or (iii) the acquisition of an infinite lifespan, often termed immortalisation. Transformation may be spontaneous or may be induced by chemical agents or viruses, and often involves a change in chromosome number.

Applications of cell and tissue culture

The main uses of animal and plant cell culture systems include:

- **Experimental model systems in biochemistry, pharmacology and physiology:** cell culture offers certain advantages over whole organism studies, with greater control over environmental conditions and biological variability. The use of genetically defined clones of cells may simplify the analysis of experimental data. Conversely, results obtained with specialised cell-based systems might be unrepresentative of a broader range of cell types and may be more difficult to interpret in terms of the whole organism.
- **Studies of the growth requirements of particular cells:** including studies of the positive effects of growth factors or growth-promoting substances, and the negative effects of xenobiotics or cytotoxic compounds. The use of cell culture in bioassays and mutagenicity testing is considered in Chapter 34.
- **Studies of cell development and differentiation:** including aspects of the cell cycle and gene expression. Cell cultures retaining their ability to differentiate *in vitro* are particularly interesting to researchers, while the lack of differentiation and unlimited growth of many animal cell lines makes them useful models of tumour development.
- **Pathological studies:** including culture of foetal cells for karyotyping and detection of genetic abnormalities, for example, trisomy.
- **Genetic manipulation:** cell culture techniques have played an essential role in the development of molecular biology, including the production of transgenic animals and plants by techniques such as transfection.
- **Biotechnology:** including the industrial production of therapeutic proteins, vaccines and monoclonal antibodies using large-scale hybridoma culture techniques similar to those used in microbiology (Chapter 34).
- **Stem cell technology:** increasingly important in biomedical research and development.

KEY POINT One of the main differences between organ and tissue incubation techniques and those used in cell/tissue culture is that the former aim simply to maintain metabolic and physiological activity for a limited period, while the latter provide conditions suitable for cell growth, division and development *in vitro* over an extended timescale, from a few days to several months.

Working with animal cell culture systems

These may be established either from whole organisms (for example, chick embryo), discrete organs (for example, rat liver) or from blood (for example, lymphocytes), typically using *mild* tissue disruption techniques, for example, enzyme treatment, where necessary.

KEY POINT Although, in theory, it is possible to culture nucleated cells from virtually any source, in practice, the highest rates of success are most often achieved with young, actively growing tissues.

SAFETY NOTE When working with tissue cultures safely, do not confuse laminar-flow cabinets with biosafety cabinets (p. 238), as they perform completely different functions. A horizontal laminar-flow hood is designed to minimise contamination of the culture rather than the worker.

SAFETY NOTE When sterilising during tissue culture (Box 41.1), take care when using 70% v/v alcohol near a naked flame (e.g. Bunsen or spirit lamp) – it is easily ignited and burns with a weakly visible flame. Make sure a fire blanket is nearby.

Example Dulbecco's modified Eagle's medium contains Ca^{2+} , Fe^{3+} , Mg^{2+} , K^+ , Na^+ , Cl^- , SO_4^{2-} , PO_4^{2-} , glucose, 20 amino acids, 10 vitamins, inositol and glutathione. Foetal calf serum is usually added at up to 20% v/v.

Using antibiotics in cell culture – a typical antimicrobial supplement might include antibacterial agents, e.g. penicillin and streptomycin, an antifungal agent, e.g. griseofulvin, and an antimycoplasmal agent, e.g. gentamicin. Use sparingly to treat contamination and discontinue once the contaminant has been eradicated.

The principal considerations in animal cell culture are:

- **Safety:** you should be aware of the potential dangers of infection from cell cultures. Although avian and rodent cells present a reduced risk of disease transmission compared with human cells, all cell cultures must be regarded as a potential source of pathogenic microbes, and appropriate sterile technique (Box 41.1) must be used at all times (see also Chapter 33). Work involving human tissues and cell cultures must be carried out in a biosafety cabinet by trained, experienced personnel – you are unlikely to gain practical experience with such cultures in the early stages of your course. For other cell cultures, you will need to follow the code of safe practice of your department – consult your departmental safety officer if you have any doubts about safe working procedures.
- **Whether to use a primary culture or a cell line:** freshly isolated cells are more likely to reflect the biochemical activities of cells *in vivo*, though they will have a limited lifespan in culture, requiring repeated isolation for longer-term projects. Continuous cell lines are more easily cultured and offer the advantage that their growth requirements in culture may be known in some detail, especially for the more widely used cell lines (for example, BHK, HeLa).
- **The requirements for a solid substratum:** some cells must be attached to a solid surface in order to grow. Anchorage dependence is a typical feature of primary cultures and finite cell lines – such cultures show density-dependent growth inhibition once the cells have formed a confluent monolayer on the surface of the substratum. An alternative approach is to grow such cells on a particulate support using 'microcarrier' beads. In contrast, many continuous cell lines can be maintained in suspension culture, as individual cells or aggregates.
- **The physico-chemical conditions,** including pH (typically 7.2–7.5) and buffering capacity, osmolality (usually 300 ± 20 mosmol kg^{-1}) and temperature (for example, 35–37 °C for mammalian cells).
- **The requirements of the culture medium:** these will include the provision of inorganic ions (as a balanced salt solution), a carbon/energy source plus other organic nutrients, and in some cases a supplement containing antimicrobial agents to counter the risks of contamination. For example, Dulbecco's modified Eagle's medium is used for many mammalian cell types that grow as adherent monolayers, while suspension cultures of continuous cell lines can be maintained using less stringent media. To support growth, the basal medium is usually supplemented with serum (usually foetal calf serum, at up to 20% v/v), or a chemically defined serum-like supplement containing a mixture of proteins, polypeptides, hormones, lipids and trace components. The *in vitro* level of CO_2 and O_2 must also be considered: many cell cultures are buffered using bicarbonate, and must be maintained in an atmosphere of elevated CO_2 , either in a sealed culture vessel or in a CO_2 incubator, to maintain pH balance.

In some cases, a pH-sensitive dye (for example, phenol red, p. 162) may be incorporated into the growth medium, to provide a visual check on pH status during growth – a colour change indicates acidification of the medium and the growth medium should be renewed.

Box 41.1 How to apply sterile technique to animal and plant cell culture

Although Chapter 33 gives general advice on the basic principles of sterile technique with microbial cultures, you will need to take additional precautions with animal and plant cell cultures to the complexity of some of the procedures and the likelihood of rapid overgrowth of any contaminating microbes. For routine work on the open bench, you should be aware of the following points:

- **Consider personal clothing and hygiene:** long hair should be tied back, or retained by a net/cap. Avoid pendulous earrings and similar items. Wash your hands at the outset, to remove any loose skin flakes: surgical gloves may be worn, though there is loss of tactile sensitivity and comfort. Hands/gloves should be swabbed with 70% v/v alcohol to further reduce the risk of contamination. If you have a cold/cough, consider wearing a face mask.
- **Work in a designated quiet area:** there should be no air currents (avoid open windows) and no 'through traffic' or other activity that might give rise to contamination (e.g. microbes should not be cultured in the same location). The term 'quiet area' also highlights that no talking should occur, to reduce the risk of aerosol contamination from the oral microflora.
- **Organise your work surface:** clear everything from the bench, swab with 70% v/v alcohol, then position all items around a central area, so you can reach every item without reaching across anything. A well-organised workspace reduces the risk of accidental contact between sterile and non-sterile items. Swab bottles and flasks with 70% v/v alcohol before positioning them at the edge of your workspace. Also, swab your work surface between procedures and at the end of the session.
- **Work close to a Bunsen flame,** positioned centrally within your workspace where convection currents create an upward airflow, reducing the likelihood of particles falling from the air into an open vessel. Always flame the tops of glass bottles, but not plastic items, for 2–3 s both *before* and *after* opening and closing, rotating the bottles during exposure to the flame. Flame glass pipettes, as described on p. 239.
- **Tilt flasks and bottles during use:** uncapped culture flasks and media bottles are best kept at a shallow angle, to minimise the risk of airborne contamination.
- **Work without delay, but do not hurry:** always keep in mind that the air contains contaminant microbes and the

longer you leave a vessel open to the air, the more likely it is that it will become contaminated.

When preparing to work in a laminar-flow cabinet, the following additional aspects should be considered:

- **Note whether the airflow is horizontal or vertical:** horizontal flow gives greatest protection to the work area and provides the most stable air flow, while vertical-flow cabinets reduce exposure of the operator. A biosafety cabinet should be used for work with potentially hazardous cultures (e.g. human cells/tissues, or with any cells known to be infected with a virus).
- **Prepare the cabinet:** switch on the cabinet and leave running for at least 10 min, then swab the work surface and other interior surfaces with 70% v/v alcohol. Swab the outsides of bottles, flasks, etc., before bringing them into the cabinet. Arrange items around a crescent-shaped central work area.
- **Carry out your work with due regard for the airflow within the cabinet:** use of a Bunsen burner is often discouraged, as it disrupts the correct airflow, creates heat and can ignite flammable items within the restricted interior of the cabinet. Always try to keep your hands/arms further away from the sterile airstream than the items within the cabinet, e.g. avoid working *behind* an open vessel in a horizontal-flow cabinet and *above* an open vessel in a vertical-flow cabinet.
- **Take particular care when using pipettes:** a laminar-flow cabinet hood will have a restricted interior volume, and it is easy for the novice to touch a pipette tip on the hood, or on another item during use. When removing a pipette from its packaging and connecting to a pipette filler (p. 140), you should point the tip of the pipette away from the user and into the air flow, holding it well above the graduated scale markings to avoid contamination. The pipette and filler are then held horizontally until required: as this can be a tricky procedure for the novice, you should not take a pipette out of its container until you are ready to use it.
- **Work carefully with open bottles and flasks:** caps can be either placed top-down on the work surface, or held in a crooked finger (Fig. 38.5(a)). Since the airflow is sterile, there is less need to work swiftly when compared to the open bench, but you should still recap all containers as soon as you have finished a particular procedure.

Using a horizontal laminar-flow cabinet – note that this is designed to minimise contamination of the culture, rather than the worker (contrast with a biosafety cabinet).

- **The equipment required:** this may include a laminar-flow or biosafety cabinet to reduce the possibility of microbial contamination, suitable culture vessels (typically pre-sterilised, disposable polystyrene dishes, bottles and flasks, treated to create a negatively charged, hydrophilic surface), a supply of high-purity water (typically distilled, deionised and carbon-filtered), a suitable incubator with temperature control of $\pm 0.5^\circ\text{C}$ or better, often with CO_2 control and mechanical mixing, and an inverted microscope to examine adherent cell monolayers during growth.

The successive stages of isolation of animal components are shown in Fig. 41.1(a). Box 41.1 gives advice on key aspects of sterile technique in cell culture; additional guidance on practical procedures in animal cell culture is given in Box 41.2.

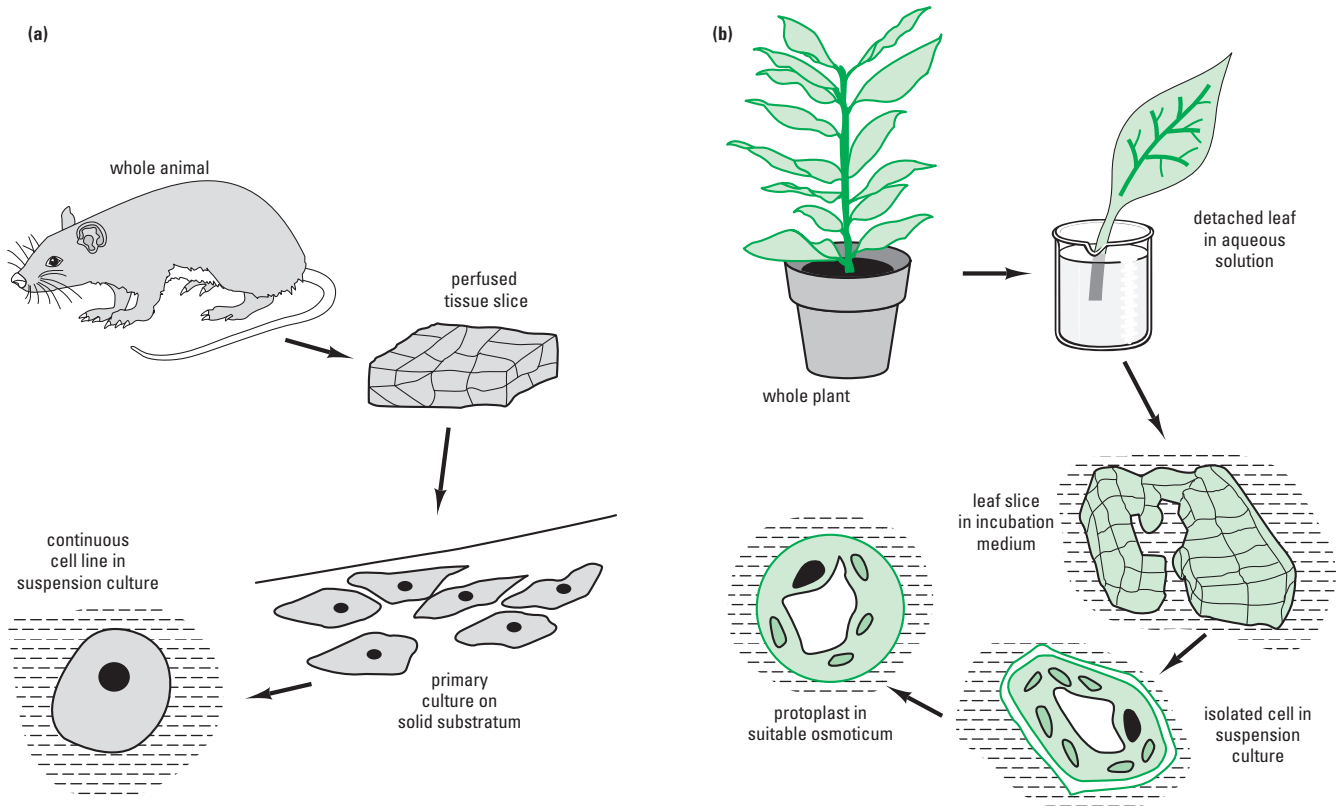


Fig. 41.1 Isolation of animal (a) and plant (b) components for *in vitro* study (note decreasing scale, from organisms to cells).

Box 41.2 How to carry out animal cell culture

The following activities are most likely to be encountered during the initial stages of your training:

- **Sterile technique (aseptic technique):** your first exercises are likely to involve transferring sterile liquids between vessels using pipettes, preparing solutions/media and making dilutions.
- **Preparing cells for initial culture:** if you are using a cryopreserved (frozen) vial containing a cell line, it will need to be thawed as rapidly as possible, to minimise the chance of intracellular ice crystal damage – thaw by immersion in water at 37 °C (if the cells were initially stored in liquid nitrogen, thaw in a lidded container as there is a risk of explosion). The thawed culture should then be diluted slowly with new growth medium. If you are preparing a primary culture, you will first have to disrupt the tissue (Chapter 42 gives further details of methods available).
- **Checking for contamination:** this can be carried out by visual inspection, looking for: (i) a cloudy medium, sometimes with a film of surface growth; (ii) a change in pH; typically a sharp decrease owing to bacterial contamination causes a colour change of the indicator dye (e.g. phenol red turns yellow). Alternatively, examine cultures microscopically, or subculture for specific microbes (Chapter 35).
- **Observing cells:** cells growing as an adherent monolayer can be observed by inverted microscope ($\times 10$ or $\times 41$ objective), looking for typical cell morphology, or cytopathic effects (e.g. rounding off and detachment, irregular shape, or internal vacuolation and granulation), as well as any evidence of microbial growth. Microscopy will also tell you when such cells are reaching confluent growth (p. 296), requiring subculture.
- **Counting cells:** while rough estimates of the growth stage and cell number may be made from direct microscopic examination of cultures, a haemocytometer is required for a fully quantitative count, as detailed in Box 34.1. Adherent cells would first be detached (e.g. using 0.25% w/v trypsin) and counted in suspension. Other quantitative approaches include electronic counting (pp. 244–6), and flow cytometry where other cellular activities can be assessed simultaneously. Alternatively, cellular constituents, e.g. DNA or protein can be assayed.
- **Checking viability:** this is usually carried out by assessing membrane integrity using either dye exclusion (1–2 min in 0.4% w/v trypan blue or 1.0% w/v naphthalene black) or, less frequently, dye uptake (10 min in 0.001% w/v diacetyl fluorescein or 0.5% w/v neutral red), with viability expressed in terms of the percentage of cells unstained or stained, respectively. When combined with haemocytometry (p. 245), the absolute numbers of live and dead cells can be estimated.
- **Feeding an adherent culture:** cells growing as a monolayer may need to have their growth medium replenished before they are ready for subculture, e.g. owing to a drop in pH of the medium, or a deterioration in cell morphology. The original medium should be removed, usually by aspiration using a sterile pipette and suction line, and a replacement volume of fresh medium then added (the new medium should be pre-warmed to 37 °C).
- **Subculturing cells ('passaging'):** typically this is carried out when adherent cells reach confluence, or when a suspension culture almost reaches the maximum population density in stationary phase. Cells growing as a monolayer must first be detached from their substrate, typically using a small amount of 0.25% w/v trypsin for up to 15 min (it is important to dilute the trypsin following detachment, to prevent further digestion). Cells in suspension culture can simply be diluted in an appropriate volume of new medium. For both types of cell culture, the reseeding density is important, since over-diluted cultures will grow poorly. For primary cultures, low dilution factors (often termed 'split ratios') of between 1:2 and 1:8 are commonly used, whereas some continuous cell lines can be diluted by up to 1:100 to give a cell density of 10^4 – 10^5 per mL.
- **Harvesting cells:** gentle centrifugation (at 80–100 *g* for 10 min) is sufficient to pellet most animal cells, leaving the spent medium as a supernatant, which should be either aspirated, or removed using a pipettor (p. 143). Monolayer cultures would be centrifuged following trypsinisation (see above).
- **Freezing cells (cryopreservation):** cells are first suspended at high density (typically around 10^6 per mL) in a suitable freezing solution, usually growth medium or serum supplemented with a cryoprotectant such as 10% v/v DMSO or 20–30% v/v glycerol, dispensed into small vials ('ampoules'/'cryotubes'), and then frozen slowly (ideally, at around 1 °C min⁻¹): one approach is to wrap the ampoules in cotton wool inside a small cardboard tube, then place them in an insulated container in a –70 °C freezer for a³ then transfer to a liquid nitrogen storage facility. An alternative approach is to use a proprietary system, e.g. 'Mr Frosty' (Nalgene), to provide a controlled rate of cooling.

Definitions

Callus – an aggregation of undifferentiated plant cells in culture.

Embryogenic callus – tissue with the capacity to differentiate under defined laboratory conditions, typically in response to plant growth regulators in the medium.

Explant – a fragment of tissue used to initiate a culture (the term is also used in animal culture).

Protoplasts – cells lacking their cell walls.

Sphaeroplasts (US, spheroplasts) – cells with attached fragments of their cell walls: osmotically sensitive.

Totipotency – the ability (of any plant cell) to de-differentiate and re-differentiate into any of the cell types found in the mature plant.

Using an orbital incubator – continued rapid agitation (>100 r.p.m.) maintains a homogeneous suspension and encourages gas exchange between the culture medium and the atmosphere.

Example Murashige and Skoog's medium contains a balanced mixture of the principal inorganic ions, plus seven trace element compounds, three vitamins, inositol, glycine and sucrose (at 30 g/L) as the major carbon source.

Working with protoplasts – remember that all solutions must contain a suitable osmoticum, to prevent bursting. Mannitol is widely used at $\approx 400 \text{ mmol L}^{-1}$.

Working with plant tissue and cell culture systems

Plant tissue cultures can be established by growing explants of sterilised tissue on the surface of an agar-based growth medium to give a callus of undifferentiated cells. Initial sterilisation is usually achieved by incubation for 15–20 min in 10% w/v sodium hypochlorite.

KEY POINT For most plants, cell cultures can be established from a broad range of tissue types, reflecting the totipotency of many plant cells.

Embryogenic callus may be induced to differentiate, forming tissues and organs on a medium containing appropriate plant hormones: in many cases, these cultures will develop to form plantlets that can be grown on to mature plants, or encapsulated to produce so-called 'artificial seeds'. This approach can be used to study the conditions necessary for differentiation and development, or to propagate rare plants and other valuable stock (for example, virus-free stock, or genetically altered plants). Callus derived from anthers can be used to provide haploid cell cultures and haploid plants – these are often useful for experimental genetics and breeding purposes.

You can obtain plant cell suspension cultures (Fig. 41.1(b)) by transferring fragments of actively growing callus to a 'shake' flask containing liquid medium in an orbital incubator: gentle agitation causes fragmentation of the callus tissue, to give a suspension culture that will contain individual plant cells and cell aggregates. This culture can be maintained by repeated subculturing of material from the upper layers of the liquid, encouraging the growth of small aggregates. However, in contrast to animal cell suspension cultures, it is rare for plant suspension cultures to be entirely unicellular. Suspension cultures often require a minimum inoculum size on subculture – an inoculum volume of 10% v/v may be necessary to ensure successful subculture. It may also be helpful to add a small amount of 'conditioned' medium from a previous culture. Commercial-scale suspension cultures are used to produce certain plant pigments and secondary metabolites such as flavourings and high-value pharmaceutical compounds.

The growth media used for callus and suspension cultures are more complex than those required for intact plant organs and tissues, with organic nutrient supplements in addition to a balanced salt solution. Such organic supplements often include a major carbon and energy source, for example, sucrose, plus various vitamins and growth regulators (typically, at least one auxin and a cytokinin), together with undefined components such as yeast extract and hydrolysed casein in some instances. Otherwise, the techniques are broadly similar to those described for microbial systems (Chapters 34 and 35).

Studying plant protoplasts

Some experimental procedures using plant cells require the enzymatic removal of their cell walls, creating protoplasts that can be manipulated *in vitro*, then maintained under conditions that allow the regeneration of cell walls, with subsequent growth and differentiation to give genetically modified plants. Protoplast isolation often involves pretreatment in a concentrated osmoticum (for example, sucrose or mannitol, at $300\text{--}500 \text{ mmol L}^{-1}$) to plasmolyse the cells, weakening the linkage between cell wall and plasma

Assessing cell and protoplast viability – cytochemical techniques are often used, e.g. exclusion of the vital stain Trypan blue or Evan's blue. The fluorogenic vital stain fluorescein diacetate is an alternative: it is cleaved by esterases within living cells, liberating fluorescein and giving green-yellow fluorescent cells when viewed by UV fluorescence microscopy.

membrane. Enzymatic treatment is often prolonged, taking several hours in a suitable mixture of enzymes, for example, cellulase and Macerozyme. The resulting material can be sieved through fine nylon mesh, centrifuged at low speed and then resuspended, to remove subcellular debris and cell aggregates. The protoplast preparation can be checked for fragments of cell wall using a suitable stain, for example, 0.1% w/v calcofluor white and a fluorescence microscope.

The fusion of protoplasts from different plants can be used to produce a somatic hybrid: this process can be used to circumvent interspecies reproductive barriers, creating novel plants. Protoplast fusion can be induced by chemical 'fusogens', for example, using polyethylene glycol (PEG) at high concentration, or by electrofusion (incubation under low alternating current to encourage aggregation, then brief exposure to a high-voltage electrical field – typically 1000 Vcm^{-1} for 1–2 ms – causing protoplast fusion). Similar techniques can be used with animal cells. The successive stages of isolation of plant components are illustrated in Fig. 41.1(b).

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STUDY EXERCISES

41.1 Calculate the osmolarity of Ringer's solution from its individual constituents. Use the data in Table 41.1 and the information in Chapter 23, and assume that each constituent behaves according to ideal thermodynamic principles. Note the following M_r values: NaCl 58.44; KCl 74.55; CaCl_2 110.99; NaHCO_3 84.01, required to convert the data in Table 41.1 to molar concentrations (see p. 153 if you are unsure about this conversion). Give your final answer in mosmol kg^{-1} to one decimal place.

41.2 Find out about the availability of cell cultures using the Web. Research the culture collections of

particular countries via their websites. How useful is each site in terms of features such as: ease of use; access to catalogues; information on individual strains, e.g. culture media; details of costs?

41.3 Research the origins of the code names used for individual cell lines. In many instances, the code names given to individual cell lines are derived from their original source. Use the Web to find out the origin of the following descriptors for cell lines:

(a) HeLa; (b) Vero; (c) BHK; (d) CHO; (e) TBV-2.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

42 Homogenising and fractionating cells and tissues

Definitions

Autolysis – breakdown of cells and tissues due to the action of internal enzymes ('self-digestion').

Disruption – a process involving structural damage to cells and tissues, to an extent where the biomolecule or complex of interest is released.

Homogenisation – a process where cells and tissues are broken into fragments small enough to create a uniform, stable emulsion (a homogenate).

Homogeniser – a general term for any equipment used to disrupt or homogenise cells and tissues.

Most biological molecules and subcellular complexes need to be isolated from their source material to be studied in detail. Unless the biomolecule is already a component of an aqueous medium (for example, plasma, tissue exudate), the first step will be to disrupt the structure of the appropriate cells or tissues. Following disruption and/or homogenisation, the *in vitro* environment of the homogenate (disrupted tissue) will be very different from that of the intact cell. Therefore, it is important to select an approach that aims to preserve the integrity of the biomolecule or subcellular complex during the isolation procedure.

KEY POINT Disruption may be achieved by chemical, physical or mechanical procedures – the choice of technique(s) required will depend on the intracellular location of the molecule and the nature of the source material.

Understanding the susceptibilities of different cell types to disruption

Cell walls are a major obstacle to disruption in many organisms and the technique used for a given application must take into account this aspect. With animal cells, there is no cell wall and the plasma membrane and cytoskeleton are relatively weak: unicellular suspensions of animal cells (for example, blood cells) can be disrupted by gentle techniques. Animal cells within tissues are more difficult to disrupt, owing to the presence of connective tissue. Muscle requires vigorous techniques, as a result of additional contractile proteins. For plant cells, the plasma membrane is surrounded by a cellulose cell wall, sometimes with additional components, for example, lignin or waxes. As a result, large shear forces are often required to disrupt plant cells.

Most Gram-positive and Gram-negative bacteria are surrounded by a rigid, protective cell wall. Peptidoglycan is a major structural component and this may be degraded by the enzyme lysozyme, particularly in certain Gram-positive organisms (Fig. 38.1(a)). In contrast, the outer membrane of Gram-negative bacteria (Fig. 38.1(b)) protects against lysozyme: however, prior treatment of Gram-negative bacteria with EDTA destabilises the outer membrane by removing Ca^{2+} , making the cell sensitive to lysozyme. In an osmotically buffered solution, the cells will be converted to sphaeroplasts (p. 301), since components of the Gram-negative envelope will remain after lysozyme treatment. Note also that some Gram-positive bacteria may be insensitive to lysozyme, owing either to a modified peptidoglycan structure or to additional protective outer layers (proteinaceous S layers: Fig. 38.1(a)).

The cell walls of filamentous fungi and yeasts are very robust, containing up to 90% polysaccharide (for example, chitin, mannan), with embedded protein microfibrils – they are often difficult to disrupt, requiring enzymic digestion and/or mechanical homogenisation. Hopkins (1991) gives further details of various systems available for homogenisation and disruption of biological material.

While disruption of both cell walls and intracellular compartments may be necessary to release biomolecules of interest, it also comes with risks, due to the release of potentially destructive enzymes (for example, proteases and nucleases) and other compounds (for example, toxins and tannins) that are contained within membrane-bound organelles such as lysosomes and vacuoles. Damage to these organelles during homogenisation will release their contents, leading to autolysis of cell components. You can reduce these effects by careful selection of the constituents of your homogenising medium.

Choosing a homogenising medium

The solution you use for homogenisation serves several purposes, since it acts as a solvent or suspension medium for the released components; it serves as a cooling medium (since many biological macromolecules are denatured by heat); and it contains various reagents that may help to preserve the biological integrity of components. A typical homogenising medium will contain:

Using buffers – selecting and preparing an appropriate buffer is covered in more detail on pp. 167–8.

Using chelating agents – EDTA (ethylenediaminetetraacetic acid) and EGTA (ethylenedis(oxyethylenetriolo)-tetraacetic acid). If Mg^{2+} is an important component of the medium, use EGTA, which does not chelate Mg^{2+} .

Extracting proteins from plants – carry out all post-homogenisation procedures as quickly as possible, because plant cell vacuoles may contain phenols that will inactivate proteins when released.

- **Buffer.** This replaces the intracellular buffer systems and is needed to prevent pH changes that might denature proteins. TRIS and phosphate buffers are often used for ‘physiological’ pH values (7.0–8.0); alternatives include various zwitterionic buffers (Chapter 24).
- **Inorganic salts.** The intracellular ionic strength is quite high, so KCl and NaCl are often included to maintain the ionic strength of the homogenate. However, the total concentration of inorganic salts should be kept below 100 mmol L^{-1} , to avoid thickening of the homogenate owing to solubilisation of structural proteins.
- **EDTA.** This chelates divalent cations and removes metal ions (for example, Cu^{2+} , Pb^{2+} , Hg^{2+}) that inactivate proteins by binding to thiol groups. In addition, it removes Ca^{2+} which could activate certain proteases, nucleases and lipases in the homogenate.
- **Sucrose.** This can be used to prevent osmotic lysis of organelles (for example, mitochondria, lysosomes), and stabilises proteins from hydrophobic intracellular environments by reducing the polarity of the aqueous medium.
- **Mg^{2+} .** This helps to preserve the integrity of membrane systems by counteracting the fixed negative charges of membrane -phospholipids.
- **Protease inhibitors** (for example, phenylmethanesulfonylfluoride, PMSF; *L-trans*-epoxysuccinylleucylamido-(4-guanodino)-butane, E-64; -leupeptin). These protect solubilised proteins from digestion by -intracellular proteases, mainly released from lysosomes on disruption of the cell. Lysosomal proteases have acid pH optima, another reason for maintaining the pH of the medium close to neutrality.
- **Reducing agents** (for example, 2-mercaptoethanol, dithiothreitol, cysteine at $\approx 1 \text{ mmol L}^{-1}$). These reagents prevent oxidation of certain proteins, particularly those with free thiol groups that may be oxidised to disulfide bonds when released from the cell under aerobic conditions.
- **Detergents** (for example, Triton X-100, SDS). These cause dissociation of proteins and lipoproteins from the cell membrane, aiding the release of membrane-bound and intracellular components.

Definitions

Hypertonic – a medium with a more negative water potential, compared with the cells or medium.

Hypotonic – a medium with a less negative water potential, compared with the cells or medium.

Isotonic – a medium with the same water potential (p. 159) as the cells or tissues.

Using osmotic shock – this approach can be effective for wall-less cells, sphaeroplasts (p. 301) and protoplasts, but is ineffective for cells with robust walls.

Disrupting cells and tissues

Prior to disruption, you will need to free animal tissues of any visible fat deposits and connective components, and then randomly slice the tissue using fine scissors or a scalpel. Remove any fibrous and vascular tissue from plant material. Disruption can be achieved either by mechanical or non-mechanical means: the principal applications of various methods are described in Table 42.1 for the major cell and tissue types.

Non-mechanical methods

- **Osmotic shock:** cells are first placed in a hypertonic solution of high osmolality (p. 155), for example 20% w/v sucrose, leading to loss of water. On dilution of this solution (for example, by addition of water or transfer to a hypotonic solution), the cells will burst because of rapid water influx.
- **Freezing and thawing:** causing leakage of intracellular material, following cell wall and membrane damage and internal disruption caused by ice crystal formation.
- **Lytic enzymes:** damaging the cell wall and/or plasma membrane. Cells can then be disrupted by osmotic shock or gentle mechanical treatment.

Table 42.1 Summary of techniques for the disruption of tissues and cells – note that safety glasses should be worn for all procedures.

Technique	Suitability	Comments
Non-mechanical methods		
Osmotic shock	Animal soft tissues; wall-less cells – protoplasts/sphaeroplasts	Small scale only
Freeze/thaw	Animal soft tissues; some bacteria	Time-consuming; small scale; closed system – suitable for pathogens with appropriate safety measures; some enzymes are cold-labile
Lytic enzymes, e.g. lipases; proteases; pectinase; cellulase	Animal cells; plant cells	Mild and selective; small scale; expensive; enzymes must be removed once lysis is complete
Lysozyme	Some bacteria	Gram-negative bacteria must be pretreated with EDTA. Suitable for some organisms resistant to mechanical disruption
Mechanical methods		
Pestle and mortar + abrasives	Tough tissues	Not suitable for delicate tissues
Ball mills + glass beads	Bacteria and fungi	May cause organelle damage in eukaryotes
Blenders and rotor-stators	Plant and animal tissues	Ineffective for microbes
Homogenisers (glass and Teflon)	Soft, delicate tissues, e.g. white blood cells, liver	Glass may shatter – wear safety glasses during use
Solid extrusion (Hughes press)	Tough plant material; bacteria; yeasts	Small scale
Liquid extrusion (French pressure cell)	Microbial cells	Small scale
Ultrasonication	Microbial cells	Cooling required; small scale; may cause damage to organelles, especially in eukaryotic cells.

Avoiding protein denaturation during homogenisation – excessive frothing of the homogenate indicates denaturation of proteins (think of whipping egg whites for meringue).

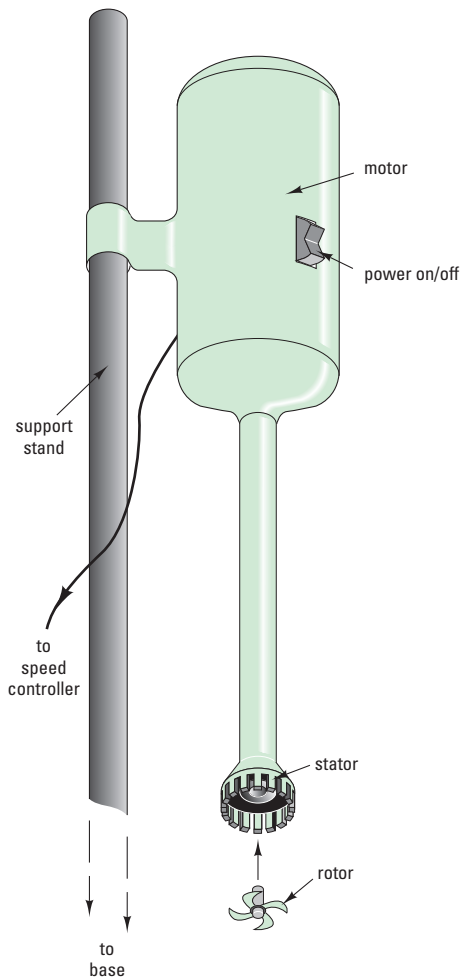


Fig. 42.1 Components of a rotor-stator homogeniser.

Mechanical methods

All mechanical procedures for cell disruption generate heat, and this may denature proteins. Therefore it is very important to cool the starting material, the homogenising medium, and, if possible, the homogeniser itself (to $\approx 4^{\circ}\text{C}$). Carry out the homogenisation in short bursts, and cool the homogenate in an ice bath between each burst. Cooling will also reduce the activity of any degradative enzymes in the homogenate. Ideally, you should carry out homogenisation in a walk-in cooler (a ‘cold room’) typically at $4\text{--}10^{\circ}\text{C}$.

Equipment commonly used includes:

- **Mixers and blenders.** These are similar to domestic liquidisers, with a static vessel and rotating blades. The Waring blender is widely used: it has a stainless steel vessel that will stay cool if pre-chilled. The vessel and blades are designed to maximise turbulence, both disrupting and homogenising tissues and cells.
- **Ball mills** (for example, Retch mixer mill, Mickle mill). These devices contain glass beads that vibrate and collide with each other and with tissues/ cells, leading to disruption.
- **Liquid extrusion devices** (for example, French pressure cell). Cells are forced from a vessel to the outside, through a very narrow orifice at high pressures ($\approx 100\text{ MPa}$). The resulting pressure changes are a powerful means of disrupting cells.
- **Solid extrusion** (for example, Hughes press). Here, a frozen cell paste is forced through a narrow orifice, where the shear forces and the abrasive properties of the ice crystals cause cell disruption.
- **Rotor-stators** (for example, IKA Ultra-turrax homogeniser). These have a rotor (a set of stainless-steel blades) and a stator (a slotted stainless steel cylinder) at the tip of a stainless steel shaft, immersed in the homogenising medium: the arrangement is illustrated in Fig. 42.1. The high speed of the rotor blades causes material in the homogenising fluid to be sucked into the dispersing head, where it is pressed radially through the slots in the stator. Along with the cutting action of the rotor blades, the material is subjected to very high shear and thrust and the resulting turbulence in the gap between rotor and stator gives effective mixing. You can adjust the vigour of the homogenisation process by varying the rotor speed setting. Various sizes of rotor-stator are available, with typical diameters in the range $8\text{--}65\text{ mm}$: the smaller sizes are particularly useful for small-scale preparations.
- **Sonicators.** Ultrasonic waves are transmitted to an aqueous suspension of cells via a metal probe. The ultrasound creates bubbles within the liquid and these produce shock waves when they collapse. Successful disruption depends on the correct choice of power and incubation time, together with pH, temperature and ionic strength of the suspension medium, often obtained by trial and error. You can reduce the effects of heating during ultrasonication by using short ‘bursts’ of power ($10\text{--}30\text{ s}$), with rests of $30\text{--}60\text{ s}$ in between, and by keeping your cell suspension on ice during disruption. An ultrasonic water bath -provides a more gentle means of disrupting certain types of cells, for example, some bacterial and animal cells.

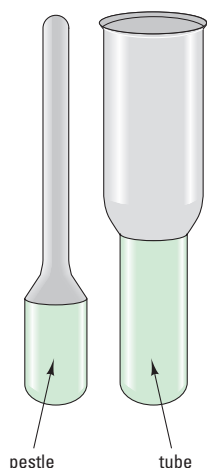


Fig. 42.2 Ground-glass homogeniser.

- **Homogenisers.** These involve the reciprocating movement of a ground-glass or Teflon pestle within a glass tube (Fig. 42.2). Cells are forced against the walls of the tube, releasing their contents. For glass pestles, the tubes also have ground-glass homogenising surfaces and may have an overflow chamber. The homogeniser can either be hand-operated (for example, Dounce), or motorised (for example, Potter-Elvehjem). Choose a ground-glass homogenizer with a suitable clearance between the pestle and the tube (range 0.05–0.5 mm) to suit your particular application.

Fractionating cells and the isolating of organelles

The fractionation and separation of organelles from a cell homogenate by differential centrifugation are described in Chapter 44. Particular organelles can be obtained by appropriate choice of source tissue and homogenisation method, as illustrated in Table 42.2 for the major types of organelle.

Table 42.2 Isolation and fractionation procedures for various organelles.

Stage	Nuclei	Mitochondria	Microsomes	Chloroplasts*
Source	Thymus tissue, which has little cytoplasm, giving high yields	Beef heart, with fat and connective tissue removed, then cubed and minced. Keep at pH 7.5 using TRIS buffer	Rat liver, stored overnight to reduce glycogen content	Spinach leaves, de-ribbed and cut into 1-cm strips
Pretreatment	Rinse with buffered physiological saline. Suspend in homogenising medium	Suspend in 2× volume of ice-cold homogenising medium. Squeeze through muslin	Chop finely with scissors and wash in 2 × volume of homogenising medium	Rinse, then suspend in 3 × volume of prechilled homogenising medium
Homogenising medium	250 mmol L ⁻¹ sucrose; 10 mmol L ⁻¹ TRIS/HCl buffer, pH 7.6; 5 mmol L ⁻¹ MgCl ₂ ; 0.2–0.5% v/v Triton X-100	250 mmol L ⁻¹ sucrose; 10 mmol L ⁻¹ TRIS/HCl buffer pH 7.7, containing 1 mmol L ⁻¹ succinic acid and 0.2 mmol L ⁻¹ EDTA	250 mmol L ⁻¹ sucrose; 50 mmol L ⁻¹ TRIS/HCl buffer, pH 7.5; 25 mmol L ⁻¹ KCl; 5 mmol L ⁻¹ MgCl ₂	400 mmol L ⁻¹ sucrose; 25 mmol L ⁻¹ HEPES/NaOH buffer at pH 7.6; 2 mmol L ⁻¹ EDTA
Homogenisation	Waring blender, low speed, 3 min	Bring to pH 7.8 using 2 mol L ⁻¹ TRIS base: Waring blender, high speed, 15 s: check and adjust pH to 7.8 and repeat blending step, 5 s	Potter-Elvehjem glass homogeniser with a Teflon pestle – 3 × 5 min at 800 rpm	Prechilled Waring blender or rotor-stator
Filtration/centrifugation	Filter through gauze. Spin at 2000 _g for 10 min; discard supernatant. Repeat the homogenisation, filtration and centrifugation stages to improve purity of organelles	Spin at 1200 <i>g</i> for 20 min: filter supernatant through muslin: centrifuge at 26 000 <i>g</i> for 15 min. Remove and discard upper (lighter) layer of pellet. Resuspend lower layer and rehomogenise (2 × 5 s). Centrifuge at 26000 <i>g</i> for 15 min	Centrifuge at 680 <i>g</i> for 10 min – discard pellet; centrifuge at 10 000 <i>g</i> for 10 min – discard pellet; centrifuge at 100000 <i>g</i> ; for 60 min – retain pellet. Resuspend in buffer, pH 8.0 and re-centrifuge at 100 000 <i>g</i> for 60 min	Pass through several layers of muslin (wear gloves); centrifuge at 2500 <i>g</i> ; for 60 s; resuspend pellet in buffer, pH 7.6; recentrifuge at 2500 <i>g</i> ; for 60 s. The colour of the supernatants gives a visual indication of chloroplast damage (e.g. if green).
Before use	Resuspend in homogenisation medium without Triton X-100	Resuspend pellet in buffer, pH 7.8 and either use immediately, or store at –20°C overnight	Resuspend in buffer solution at pH 8.0	Resuspend pellet in appropriate incubation medium, containing sucrose, e.g. for CO ₂ /O ₂ studies, p. 430

*An alternative approach is to use plant protoplasts (pp. 301–2) as the starting material, releasing the chloroplasts by gentle lysis – diluting the medium with water.

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STUDY EXERCISES

42.1 Consider suitable disruption procedures for particular types of cell or tissue. After reading this chapter, identify the major problems associated with disrupting each of the following, and list suitable practical procedures to overcome these problems:

- (a) Gram-negative bacteria;
- (b) skeletal muscle;
- (c) plant tissue.

42.2 Select a suitable disruption technique for the preparation of intracellular components. How might the procedure used to prepare a soluble cytoplasmic protein from fresh liver differ from that required for the isolation of intact organelles?

42.3 Test your understanding of the function of homogenisation medium components. The following list provides a number of possible

components that might be used in a homogenisation medium:

- (a) sucrose;
- (b) low ionic strength buffer;
- (c) dithiothreitol;
- (d) detergent (for example, Triton X-100).

Select one of these components to include in a medium for preparing:

- (i) a membrane protein from liver tissue;
- (ii) a mitochondrial suspension from liver tissue;
- (iii) an oxygen-sensitive enzyme from pancreatic tissue;
- (iv) a protein from the sarcolemma (cytoplasm) of skeletal muscle.

Give a brief explanation for your selection in each case.

Answers to these study exercises are available at go.pearson.com/uk/he/resources



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43 Understanding calibration and quantitative analysis

Understanding quantitative measurement

– Chapter 27 contains details of the basic principles of valid measurement, while Chapters 45–55 deal with some of the specific analytical techniques used in biology. The use of internal standards is covered on p. 362.

Calibrating laboratory apparatus – this is important in relation to validation of equipment, e.g. when determining the accuracy and precision of a pipettor by the weighing method: see pp. 143–4.

There are many instances where it is necessary to measure the quantity of a test substance using a calibrated procedure. You are most likely to encounter this approach in one or more of the following practical exercises:

- **quantitative spectrophotometric assay of biomolecules** (Chapter 46)
- **flame or atomic absorption spectroscopic analysis** of metal ions in biological solutions (pp. 344–345)
- **using a chromogenic or fluorogenic substrate** to determine the activity of an enzyme
- **quantitative chromatographic analysis**, for example, GC or HPLC (Chapter 48)
- **using a bioassay system** to quantify a test substance: examples include immunodiffusion (pp. 393, 394) and radioimmunoassay (pp. 395–6).

KEY POINT In most instances, calibration involves the establishment of a relationship between the measured response (the 'signal') and one or more 'standards' containing a known amount of substance.

In some instances, you can measure a signal due to an inherent property of the substance, for example, the absorption of UV light by nucleic acids (pp. 488–9), whereas in other cases you will need to react it with another substance to see the result (for example, molecular weight measurement of DNA fragments after electrophoresis, visualised using SYBR Safe, p. 515), or to produce a measurable response (for example, the reaction of cupric ions and peptide bonds in the Biuret assay for proteins, p. 426).

Describing the different types of calibration curve

By preparing a set of solutions (termed 'standards'), each containing either (i) a known *amount* or (ii) a specific *concentration* of the substance, and then measuring the response of each standard solution, the underlying relationship can be established in graphical form as a 'calibration curve', or 'standard curve'. This can then be used to determine either (i) the amount or (ii) the concentration of the substance in one or more test samples. Alternatively, the response can be expressed solely in mathematical terms: an example of this approach is the determination of chlorophyll pigments in plant extracts by measuring absorption at particular wavelengths, and then applying a formula based on previous (published) measurements for purified pigments.

There are various types of standard curve: in the simplest cases, the relationship between signal and substance will be linear, or nearly so, and the calibration will be represented best by a straight-line graph (see Box 43.1). In some instances (Fig. 43.1(a)), you will need to transform either the x values or y values (Chapter 71), to produce a linear graph (for example, in radial immunodiffusion bioassay, where the y values are squared). In other instances, the straight-line relationship may only hold up to a certain value (the 'linear dynamic range') and beyond this point the graph may curve (for

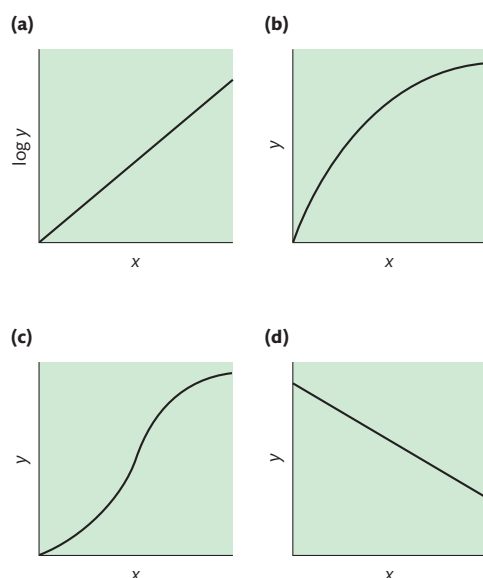


Fig. 43.1 Calibration curves: (a) log-linear; (b) curvilinear; (c) sigmoid, or S-shaped; (d) inverse.

Box 43.1 How to prepare and use a calibration curve

- 1. Decide on an appropriate test method** – for example, in a project, you may need to research the best approach to the analysis of a particular metabolite in your biological material.
- 2. Select either (a) amount or (b) concentration, and an appropriate range and number of standards** – in practical classes, this may be given in your schedule, along with detailed instructions on how to make up the standard solutions. In other cases, you may be expected to work this out from first principles (Chapters 22 and 23 give worked examples) – aim to have evenly spaced values along the x axis.
- 3. Prepare your standards very carefully** – due attention to detail is required: for example, you should ensure that you check the calibration of pipettors beforehand, using the weighing method. Do not forget the ‘zero standard’ plus any other controls required, e.g. to test for interference due to other chemical substances. Your standards should cover the range of values expected in your test samples.
- 4. Assay the standards and the unknown (test) samples** – preferably all at the same time, to avoid introducing error due to changes in the sensitivity or drift in the zero setting of the instrument with time. It is a good idea to measure all of your standard solutions at the outset, and then measure your test solutions, checking that the ‘zero standard’ and ‘top standard’ give the same values after, say, every six test measurements. If the remeasured standards do not fall within a reasonable margin of the previous value, then you will have to go back and recalibrate the instrument, and repeat the last six test measurements. If your test samples lie outside the range of your standards, you may need to repeat the assay using diluted test samples (extrapolation of your curve may not be valid).
- 5. Draw the standard curve, or determine the underlying relationship** – Figure 43.2 gives an example of a typical linear calibration curve, where the spectrophotometric absorbance of a series of standard solutions is related to the amount of substance. When using a spreadsheet (Box 43.2) or graphing software (Chapter 74), it is often

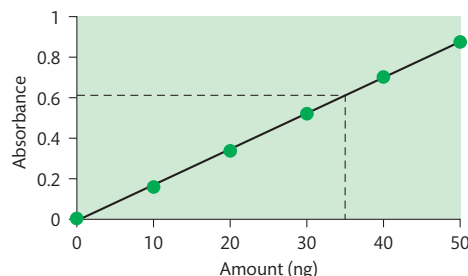


Fig. 43.2 Typical calibration curve for spectrophotometric analysis.

appropriate to use Model I linear regression to produce a linear trend line (also termed the ‘line of best fit’) and you can then quote the value of r^2 , which is a measure of the ‘fit’ of the measurements to the line. However, you should take care not to use a linear plot when the underlying relationship is clearly nonlinear (Fig. 77.7(b)) and you must consider whether the assumptions of the regression analysis are valid (e.g. for transformed data, Chapter 71).

- 6. Determine the amount or concentration in each unknown sample** – either by reading the appropriate value from the calibration curve, or by using the underlying mathematical relationship, i.e. $y = a + bx$ (pp. 587–8). Make sure you draw any horizontal and vertical construction lines very carefully – students often lose marks unnecessarily by submitting poorly drawn construction lines within practical reports.
- 7. Correct for dilution or concentration, where appropriate** – for example, if you diluted each test sample by ten-fold, then you would need to multiply by 10 to determine the value for the undiluted test sample. As another example, if you assayed 0.2 mL of test sample, you would need to multiply the value obtained from the calibration curve by 5, to give the value per mL.
- 8. Quote your test results to an appropriate number of significant figures** – this should reflect the accuracy of the method used not the size of your calculator’s display.

example, in quantitative spectrophotometry, the Beer–Lambert relationship often becomes invalid at high absorbance, giving a curve, Fig. 43.1(b)). Some calibration curves are sigmoid (Fig. 43.1(c)), in which case the central near-linear part should be used, or a suitable process used to create a mathematical relationship that describes the curve. Finally, the signal may *decrease* in response to an increase in the substance (Fig. 43.1(d)), for

Example A test tube containing 8 mL of water plus 2 mL of 1% w/v NaCl ($M_r = 58.44$) would have a mass concentration of 0.2% w/v NaCl (a five-fold dilution of the original NaCl solution), which can also be expressed as 2 g L^{-1} ; in terms of molar concentration (p. 146), this would be equivalent to $2 \div 58.44 = 0.0342 \text{ mol L}^{-1}$ (to three significant figures). Expressed in terms of the *amount* of NaCl in the test tube, this would be 0.02 g in mass, or $0.02 \div 58.44 = 3.42 \times 10^{-4} \text{ mol}$ (342 μmol) in moles.

Consulting national/ international standards – organisations providing these include:

- Laboratory of the Government Chemist (<https://www.lgc.co.uk/>), for UK standards and European Reference Materials.
- National Institute for Science and Technology (<https://www.cstl.nist.gov/>), for biochemical/chemical standards in the USA.
- Institute for Reference Materials and Measurements (<https://www.irmm.jrc.be/>), for European standards, including BCR and ERM materials.
- OIE Biological Standards Commission (follow the tab for 'Standard Setting' at <http://www.oie.int>), for international animal standards.

Plotting a standard curve – do not force your calibration line to pass through zero if it clearly does not. There is no reason to assume that the zero value is any more accurate than any other reading you have made.

example, radioimmunoassay, where an inverse sigmoid calibration curve is obtained. In some practical classes, you may be told that the relationship is expected to be linear, curvilinear, or whatever, while in others you may be expected to decide the form of the standard curve as part of the exercise.

Carrying out a calibration

Choosing whether to use amount or concentration

This first step is often the most confusing for new students. It is vital that you understand the difference between *amount* of substance (for example, mg, ng, etc.), and *concentration* (the amount of substance per unit volume, for example, mmol L^{-1} , mol m^{-3} , % w/v, etc.) before you begin your practical work.

KEY POINT Essentially, you have to choose whether to work in terms of either (i) the total amount of substance in your assay vessel (e.g. test tube, or cuvette) or (ii) the final concentration of the substance in your assay vessel.

The interconversion of amount and concentration is covered in more detail on. Either way, this is usually plotted on the x (horizontal) axis and the measured response on the y (vertical) axis.

Deciding on the standards to use

In your early practical classes, you may be provided with a stock solution (p. 146), from which you then have to prepare a specified number of standard solutions. In such cases, you will need to understand how to use dilutions to achieve the required amounts or concentrations. In later work and projects, you may need to prepare your standards from chemical reagents in solid form, where the important considerations are purity and solubility. For professional analysis (for example, in forensic science or clinical biochemistry), it is often important to be able to trace the original standard or stock solution back to national or international standards or to certified reference materials.

Deciding how many standards you need

This may be given in your practical schedule, or you may have to decide what is appropriate (for example, in project work and research). If the form and working range of the standard curve are known in advance, these may influence your choice – for example, linear calibration curves can be established with fewer standards than curvilinear relationships. In some instances, analytical instruments can be calibrated using a single standard solution, often termed a 'single point calibrator'. Replication of each standard solution is a good idea, since it will give you some information on the variability involved in preparing and assaying the standards. Consider whether you should plot mean values on your standard curve, or whether it is better to plot the individual values (if one value appears to be well off the line, you have made an error, and you may need to check and repeat the measurement).

Box 43.2 How to use a spreadsheet (e.g. Microsoft Excel) to produce a linear regression plot

Two approaches are possible: either using the *Trendline* feature or using the regression analysis function within the *Data analysis* tool pack. Both make the assumption that the criteria for Model I linear regression are met. In the example shown below, the following simple data set has been used:

Amount (ng)	Absorbance
0	0.00
10	0.19
20	0.37
30	0.56
40	0.63
50	0.78

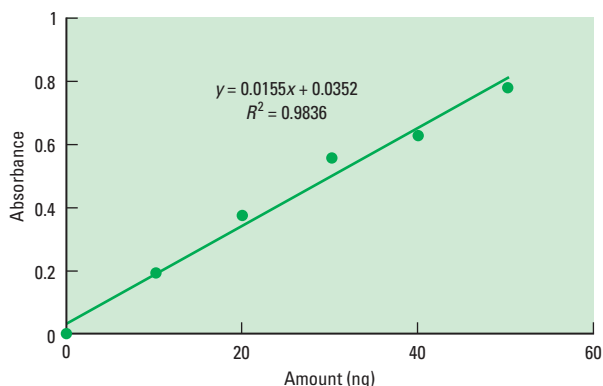


Fig. 43.3 Calibration curve showing line of best fit and details of linear regression equation.

A. Using the *Trendline* feature

This quick method provides a line of best fit on an *Excel* chart and can also provide a set of equation values for predictive purposes.

- 1. Create a graph (chart) of your data.** Enter the data in two columns within your spreadsheet, select the data array (highlight using left mouse button) and then, using the *Chart Wizard* icon, select *Chart type* > *XY (Scatter)*.
- 2. Add a trendline.** Right-click on any of the data points on your graph, and select the *Add Trendline* menu. Choose the *Linear* trendline option, but do not click *OK* at this stage. Rather, from the *Options* menu, select: (i) *Display equation on chart* and (ii) *Display R-squared value on chart*. Now click *OK*. The equation (shown in the form $y = bx + a$) gives the slope and intercept of the line of best fit, while the R-squared value (coefficient of determination, pp. 613–14) gives the proportional fit to the line (the closer this value is to 1, the better the fit of the data to the trendline).
- 3. Modify the graph to improve its effectiveness.** For a graph that is to be used elsewhere (e.g. in a lab write-up or project report), adjust the display to remove the default background and gridlines and change the symbol shape (see Box 77.2 for more advice and examples). Right-click on the trend line and use the *Format Trendline* menu to adjust the *Line Style* > *Width* of the line to make it thinner. Drag and move the equation panel if you would

like to alter its location on the chart, or delete it, having noted the values. Figure 43.3 shows a calibration curve produced in this way for the data presented above.

- 4. Use the regression equation to estimate unknown (test samples).** By rearranging the equation for a straight line and substituting a particular *y*-value, you can predict the amount/concentration of substance (*x*-value) in a test sample. This is more precise than simply reading the values from the graph using construction lines (Box 43.1). If you are carrying out multiple calculations, the appropriate equation, $x = (y - a)/b$, can be entered into a spreadsheet, for convenience.

B. Using the regression analysis tool

This requires the 'data analysis tool pack' to be loaded beforehand (using *Excel Options* under the Microsoft *Office* button in the top left corner, then select *Add-Ins* > *Analysis ToolPak*) and provides summary output that contains details of slope, intercept and coefficient of determination along with an analysis of variance (ANOVA) table (Chapter 77 gives further details of these aspects, including an example of output in Study exercise 77.4).

Note: instructions illustrated here may vary among the different versions of Microsoft *Office* Programs. Check the exact functions and syntax using the *Insert function* menu option (fx button in some versions).

Dealing with interfering substances – one approach is to use the method of ‘standard additions’, where the standards all contain a fixed additional amount of the sample (for more detail of this approach, see e.g. Dean, 1997). Internal standards (p. 362) can also be used to detect such problems.

Preparing your standards

It is extremely important to take the greatest care to measure out all chemicals and liquids very accurately, to achieve the best possible standard curve. The grade of volumetric flask used and temperature of the solution also affect accuracy (grade A apparatus is best). You may also consider what other additives might be required in your standard solutions. For example, do your test samples have high levels of potentially interfering substances, and should these also be added to your standards? Also consider what controls and blank solutions to prepare.

KEY POINT The validity of your standard curve depends upon careful preparation of standards, especially in relation to accurate dispensing of the volumes of any stock solution and the diluting liquid (diluent) – the results for your test samples can only be as good as your standard curve.

Generating the calibration curve and estimating the amount of the unknown (test) sample(s)

This is described in stepwise fashion within Box 43.1. Check you understand the requirements of graph drawing, especially in relation to plotted curves and the mathematics of straight-line graphs. Spreadsheet programs such as Microsoft *Excel* can be used to produce a regression line for a straight-line calibration plot. Examples of how to do this are provided in Box 43.2.

Text reference

Dean, J.D. (1997) *Atomic Absorption and Plasma Spectroscopy*, 2nd edn. Wiley, Chichester. [Chapter 1 deals with calibration, and covers the principle of standard additions.]

Sources for further study

Cable, M. (2005) *Calibration: A Technician's Guide*. ISA, Raleigh.

Harris, D.C. (2020) *Quantitative Chemical Analysis*, 10th edn. Freeman, New York.

Miller, J.N. and Miller, J.C. and Miller, R.D. (2018) *Statistics and Chemometrics for Analytical Chemistry*, 7th edn. Pearson Education, Harlow. [Gives detailed coverage of calibration methods and the validity of analytical measurements.]

STUDY EXERCISES

43.1 Determine unknowns from a hand-drawn calibration curve. The following data are for a set of calibration standards for Zn, measured by atomic absorption spectrophotometry.

Absorbance measurements for a series of standard solutions containing different amounts of zinc

Zinc concentration ($\mu\text{g mL}^{-1}$)	Absorbance
0	0.000
1	0.082
2	0.174
3	0.257
4	0.340
5	0.408
6	0.463
7	0.511
8	0.543
9	0.561
10	0.575

Draw a calibration curve by hand using graph paper and estimate the concentration of zinc in the following water samples:

- an undiluted sample, giving an absorbance of 0.157
- a twenty-fold dilution, giving an absorbance of 0.304
- a five-fold dilution, giving an absorbance of 0.550.

Give your answer to three significant figures in each case.

43.2 Determine unknowns from a calibration curve produced in Excel. The following data are for a set of calibration standards for protein content, measured by the Lowry (Folin-Ciocalteu) method (p. 426).

Absorbance measurements for a series of protein standards

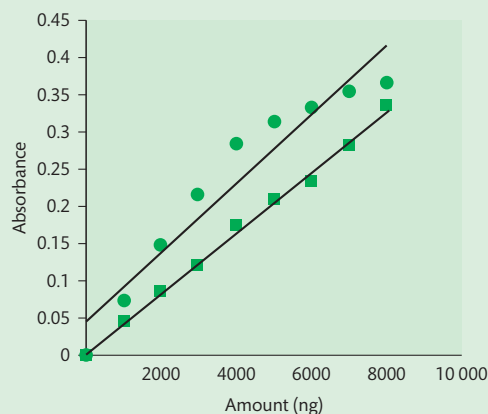
Protein (mg per tube)	Absorbance
0.00	0.000
0.02	0.161
0.04	0.284
0.06	0.438
0.08	0.572
0.10	0.762

Using PC-based software (e.g. *Excel*), fit a trend line (linear regression) and determine the protein content of solutions with the following absorbances:

- 0.225
- 0.465
- 0.682

Give your answers to three significant figures in each case.

43.3 Identify the errors in a calibration curve. The figure shows a calibration curve of the type that might be submitted in a practical write-up. List the errors and compare your observations with the list given on the Website.



Calibration graph

Answers to these study exercises are available at go.pearson.com/uk/he/resources

44 Centrifugation techniques

SAFETY NOTE Working with centrifuges – these instruments generate very high centrifugal forces and should only be used after suitable instruction and training. *If in doubt, ask.*

Table 44.1 Relationship between speed (r.p.m.) and acceleration (relative centrifugal field, RCF) for a typical bench centrifuge with an average radius of rotation, $r_{av} = 115$ mm

r.p.m.	RCF*
500	30
1000	130
1500	290
2000	510
2500	800
3000	1160
3500	1570
4000	2060
4500	2600
5000	3210
5500	3890
6000	4630

*RCF values rounded to nearest 10.

Particles suspended in a liquid will move at a rate that depends on:

- **the applied force** – particles in a liquid within a gravitational field, for example, a stationary test tube, will move in response to the earth's gravity
- **the density difference between the particles and the liquid** – particles less dense than the liquid will float upwards while particles denser than the liquid will sink
- **the size and shape of the particles**
- **the viscosity of the medium.**

For most biological particles (cells, organelles or molecules) the rate of flotation or sedimentation in response to the earth's gravity is too slow to be of practical use in separation. For this reason, centrifuges are used to speed up the process.

KEY POINT A centrifuge is an instrument designed to produce a centrifugal force far greater than the earth's gravity, by spinning the sample about a central axis (Fig. 44.1 and Table 44.1). Particles of different size, shape or density will thereby sediment at different rates, depending on the speed of rotation and their distance from the central axis.

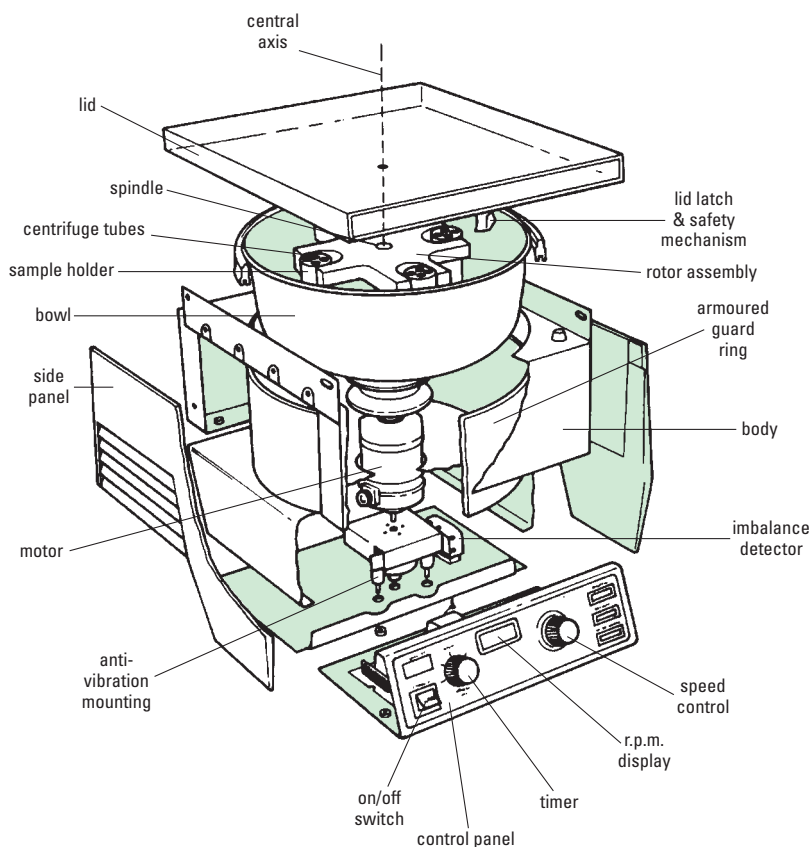


Fig. 44.1 Principal components of a low-speed bench centrifuge. Adapted from diagram of low-speed centrifuge model MSE Centaur 2, supplied by Fisher Scientific UK Ltd.

Working in SI units – to convert RCF to acceleration in SI units, multiply by 9.80 ms^{-2} :
e.g. an RCF of 290 is equivalent to an acceleration of $290 \times 9.80 = 2842 \text{ ms}^{-2}$.

Examples Suppose you wanted to calculate the RCF of a bench centrifuge with a rotor of $r_{av} = 95 \text{ mm}$ running at a speed of 3000 r.p.m. Using eqn [44.1] the RCF would be: $1.118 \times 95 \times (3)^2 = 956g$.

You might wish to calculate the speed (r.p.m.) required to produce a relative centrifugal field of 2000 g using a rotor of $r_{av} = 85 \text{ mm}$. Using eqn [44.2] the speed would be: $945.7 \sqrt{(2000 \div 85)} = 4587 \text{ r.p.m.}$

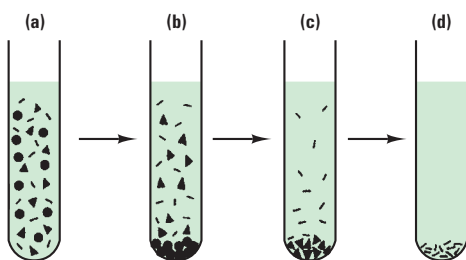


Fig. 44.2 Differential sedimentation. (a) Before centrifugation, the tube contains a mixed suspension of large, medium and small particles of similar density. (b) After low-speed centrifugation, the pellet is predominantly composed of the largest particles. (c) Further high-speed centrifugation of the supernatant will give a second pellet, predominantly composed of medium-sized particles. (d) A final ultracentrifugation step pellets the remaining small particles. Note that all of the pellets apart from the final one will have some degree of cross-contamination.

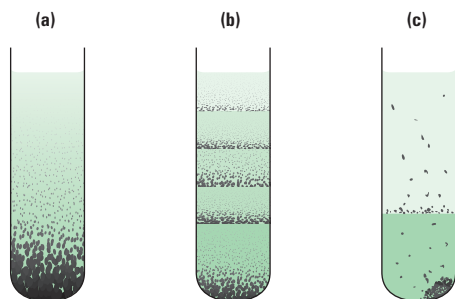


Fig. 44.3 Density gradients. (a) A continuous (linear) density gradient. (b) A discontinuous (stepwise) density gradient, formed by layering solutions of decreasing density on top of each other. (c) A single-step density barrier, designed to allow selective sedimentation of one type of particle.

Calculating centrifugal acceleration

The acceleration of a centrifuge is usually expressed as a multiple of the acceleration due to gravity ($g = 9.80 \text{ ms}^{-2}$), termed the relative centrifugal field (RCF, or ‘ g value’). The RCF depends on the speed of the rotor (n , in revolutions per minute, r.p.m.) and the radius of rotation (r , in mm) where:

$$\text{RCF} = 1.118r \left(\frac{n}{1000} \right)^2 \quad [44.1]$$

This relationship can be rearranged, to calculate the speed (r.p.m.) for specific values of r and RCF:

$$n = 945.7 \sqrt{\left(\frac{\text{RCF}}{r} \right)} \quad [44.2]$$

However, you should note that RCF is not uniform within a centrifuge tube: it is highest near the outside of the rotor (r_{\max}) and lowest near the central axis (r_{\min}). In practice, it is customary to report the RCF calculated from the average radius of rotation (r_{av}), as shown in Fig. 44.5 below. It is also worth noting that RCF varies relative to the *square* of the speed: thus the RCF will be doubled by an increase in speed of approximately 41% (Table 44.1).

Understanding centrifugal separation methods

Differential sedimentation (pelleting)

By centrifuging a mixed suspension of particles at a specific RCF for a particular time, the mixture will be separated into a pellet and a supernatant (Fig. 44.2). The successive pelleting of a suspension by spinning for a fixed time at increasing RCF is widely used to separate organelles from cell homogenates. The same principle applies when cells are harvested from a liquid medium.

Density-gradient centrifugation

The following techniques use a density gradient, a solution that increases in density from the top to the bottom of a centrifuge tube (Fig. 44.3).

- **Rate-zonal centrifugation.** By layering a sample on to a shallow pre-formed density gradient, followed by centrifugation, the larger particles will move faster through the gradient than the smaller ones, forming several distinct zones (bands). This method is time-dependent, and centrifugation *must* be stopped before any band reaches the bottom of the tube (Fig. 44.4).
- **Isopycnic centrifugation.** This technique separates particles on the basis of their buoyant density. Several substances form density gradients during centrifugation (for example, sucrose, CsCl, Ficoll, Percoll, Nycodenz). The sample is mixed with the appropriate substance and then centrifuged – particles form bands where their density corresponds to that of the medium (Fig. 44.4). This method requires a steep gradient and sufficient time to allow gradient formation and particle redistribution, but is unaffected by further centrifugation.

Working with silicone oil – the density of silicone oil is temperature-sensitive, so you should work in a location with a known, stable temperature or the technique may fail.

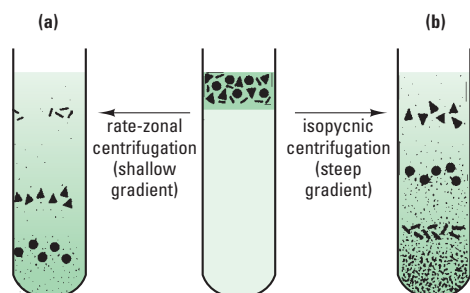


Fig. 44.4 Density-gradient centrifugation. The central tube shows the position of the sample prior to centrifugation, as a layer on top of the density gradient medium. Note that particles sediment on the basis of size during rate-zonal centrifugation (a), but form bands in order of their densities during isopycnic centrifugation (b). ●, large particles, intermediate density; ▲, medium-sized particles, low density; ■, small particles, high density.

Recording usage of high-speed centrifuges and ultracentrifuges – most departments have a log book (for samples/speeds/times): make sure you record these details, as the information is important for servicing and replacement of rotors.

Bands within a density gradient can be sampled using a fine Pasteur pipette, or a syringe with a long, fine needle. Alternatively, the tube may be punctured and the contents (fractions) collected dropwise in several tubes. For accurate work, an upward displacement technique can be used (see Ford and Graham, 1991).

Density-barrier centrifugation

A single-step density barrier (Fig. 44.3(c)) can be used to separate cells from their surrounding fluid, for example, using a layer of silicone oil adjusted to the correct density using dinonyl phthalate. Blood cell types can be separated using a density barrier of, for example, Ficoll.

Choosing an appropriate type of centrifuge

Low-speed centrifuges

These are bench-top instruments for routine use, with a maximum speed of 3000–6000 r.p.m. and RCF up to 6000 *g* (Fig. 44.1). Choose this type when harvesting cells, larger organelles (for example, nuclei, chloroplasts) and coarse precipitates (for example, antibody–antigen complexes). Most modern machines also have a sensor that detects any imbalance when the rotor is spinning and cuts off the power supply (Fig. 44.1). However, some of the older models do not, and must be switched off as soon as any vibration is noticed, to prevent damage to the rotor or harm to the operator. Box 44.1 gives details of operation for a low-speed centrifuge.

Microcentrifuges (microfuges)

These are bench-top machines, capable of rapid acceleration up to 12 000 r.p.m. and 10 000 *g*. Select this type when you are required to sediment small sample volumes (up to 1.5 mL) of larger particles (for example, cells, precipitates) over short timescales (typically, 0.5–15 min). They are particularly useful for the rapid separation of cells from a liquid medium, for example, silicone oil microcentrifugation.

Continuous-flow centrifuges

You can use this type to harvest large volumes of cells from their growth medium. During centrifugation, the particles are sedimented as the liquid flows through the rotor.

High-speed centrifuges

These are usually larger, free-standing instruments with a maximum speed of up to 25 000 r.p.m. and RCF up to 60 000 *g*. Choose for isolating microbial cells, larger organelles (for example, mitochondria, lysosomes) and protein precipitates. They often have a refrigeration system to keep the rotor cool at high speed. You would normally use such instruments only under direct supervision.

Ultracentrifuges

These are the most powerful machines, having maximum speeds in excess of 30 000 r.p.m. and RCF up to 600 000 *g*, with sophisticated refrigeration and vacuum systems. Select when isolating smaller organelles (for example, ribosomes, membrane vesicles) and biological macromolecules. You would not normally use an ultracentrifuge, though your samples may be run by a member of staff.

SAFETY NOTE When changing a rotor, make sure that you carry it properly (do not knock/drop it), that you fit it correctly (do not cross-thread it, and tighten to the correct setting using a torque wrench) and that you store it correctly (clean it after use and do not leave it lying around).

Selecting a rotor

Many centrifuges can be used with tubes of different size and capacity, either by changing the rotor, or by using a single rotor with different buckets/adaptors. You can choose between the following:

- **Swing-out rotors:** sample tubes are placed in buckets that pivot as the rotor accelerates (Fig. 44.5(a)). Swing-out rotors are used on many low-speed centrifuges: their major drawback is their extended path length and the resuspension of pellets due to currents created during deceleration.
- **Fixed-angle rotors:** used in many high-speed centrifuges and microcentrifuges (Fig. 44.5(b)). With their shorter path length, fixed rotors are more effective at pelleting particles than swing-out rotors.
- **Vertical-tube rotors:** used for isopycnic density-gradient centrifugation in high-speed centrifuges and ultracentrifuges (Fig. 44.5(c)). They cannot be used to harvest particles in suspension as a pellet is not formed.

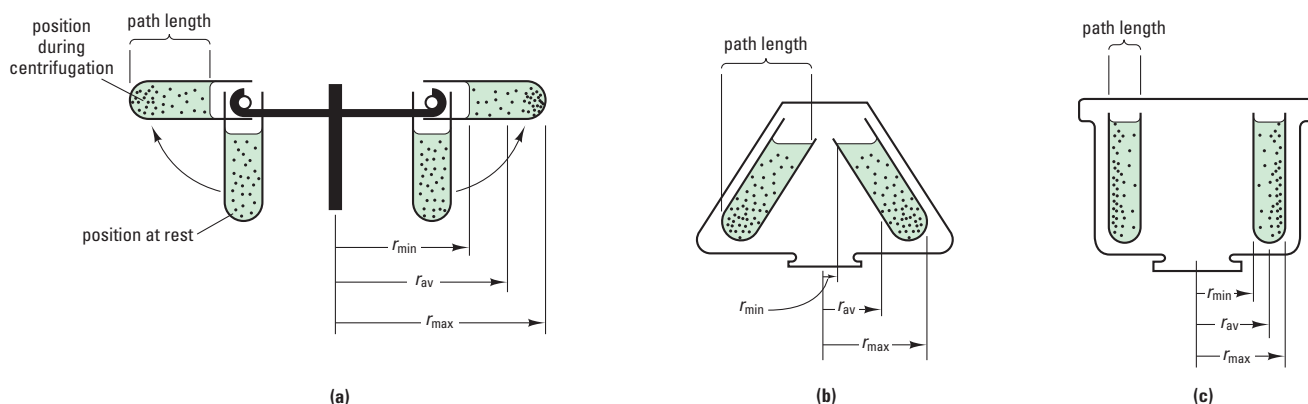


Fig. 44.5 Rotors: (a) swing-out rotor; (b) fixed-angle rotor; (c) vertical-tube rotor.

Choosing centrifuge tubes

These are manufactured in a range of sizes (from 1.5 mL up to 1000 mL) and materials. The following aspects may influence your choice:

SAFETY NOTE When working with centrifuge tubes, *never* be tempted to use a tube or bottle that was not designed to fit the machine you are using (e.g. a general-purpose glass test tube, or a screw-capped bottle), or you may damage the centrifuge and cause an accident.

Using microcentrifuge tubes – the integral push-on caps of microcentrifuge tubes must be correctly pushed home before use or they may come off during centrifugation.

- **Capacity.** This is obviously governed by the volume of your sample. Note that centrifuge tubes must be completely full for certain applications, for example, for high-speed work.
- **Shape.** Conical-bottomed centrifuge tubes retain pellets more effectively than round-bottomed tubes, while the latter may be more useful for density-gradient work.
- **Maximum centrifugal force.** Detailed information is supplied by the manufacturers. Standard Pyrex glass tubes can only be used at low centrifugal force (up to 2000 g).
- **Caps.** Most fixed-angle and vertical-tube rotors require tubes to be capped, to prevent leakage during use and to provide support to the tube during centrifugation. For low-speed centrifugation, caps must be used for any hazardous samples. Make sure you use the correct caps for your tubes.

Box 44.1 How to use a low-speed bench centrifuge

1. **Choose the appropriate tube size and material for your application**, with caps where necessary. Most low-speed machines have four-place or six-place rotors – use the correct number of samples to *fill* the rotor assembly whenever possible.
2. **Fill the containers to the appropriate level**: do not overfill, or the sample may spill during centrifugation.
3. **It is vital that the rotor is balanced during use**. Therefore, *identical* tubes must be prepared, to be placed opposite each other in the rotor assembly. This is particularly important for density-gradient samples, or for samples containing materials of widely differing densities, e.g. soil samples, since the density profile of the tube will change during a run. However, for low-speed work using small amounts of particulate matter in aqueous solution, it is sufficient to counterbalance a sample with a second tube filled with water, or a saline solution of similar density to the sample.
4. **Balance each pair of sample tubes** (plus the corresponding caps, where necessary) to within 0.1 g using a top-pan balance; add liquid dropwise to the lighter tube, until the desired weight is reached. Alternatively, use a set of scales. For small sample volumes (up to 10 mL) added to disposable, lightweight plastic tubes, accurate pipetting of your solution may be sufficient for low-speed use.
5. **For centrifuges with swing-out rotors**, check that each holder/bucket is correctly positioned in its locating slots on the rotor and that it is able to swing freely. All buckets must be in position on a swing-out rotor, even if they do not contain sample tubes – buckets are an integral part of the rotor assembly.
6. **Load the sample tubes into the centrifuge**. Make sure that the outside of the centrifuge tubes, the sample holders and sample chambers are dry: any liquid present will cause an imbalance during centrifugation, in addition to the corrosive damage it may cause to the rotor. For sample holders where rubber cushions are provided, make sure that these are correctly located. Balanced tubes must be placed opposite each other – use a simple code if necessary, to prevent mix-ups.
7. **Bring the centrifuge up to operating speed** by gentle acceleration. Do not exceed the maximum speed for the rotor and tubes used.
8. **If the centrifuge vibrates at any time during use, switch off** and find the source of the problem.
9. **Once the rotor has stopped spinning, release the lid and remove all tubes**. If any sample has spilled, make sure you clean it up thoroughly using a noncorrosive disinfectant, e.g. Virkon, so that it is ready for the next user.
10. **Close the lid (to prevent the entry of dust) and return all controls to zero**.

- **Solvent resistance**. Glass tubes are inert, polycarbonate tubes are particularly sensitive to organic solvents (for example, ethanol, acetone), while polypropylene tubes are more resistant. See manufacturer's guidelines for detailed information.
- **Sterility**. Disposable plastic centrifuge tubes are often supplied in sterile form. Glass and polypropylene tubes can be repeatedly sterilised. Cellulose ester tubes should *not* be autoclaved. Repeated autoclaving of polycarbonate tubes may lead to cracking/stress damage.
- **Opacity**. Glass and polycarbonate tubes are clear, while polypropylene tubes are more opaque.
- **Ability to be pierced**. If you intend to harvest your sample by puncturing the tube wall, cellulose acetate and polypropylene tubes are readily punctured using a syringe needle.

SAFETY NOTE Never balance centrifuge tubes 'by eye' – use a balance. Note that a 35 mL tube full of liquid at an RCF of 3000 *g* has an effective weight greater than a large adult man.

Balancing the rotor

For the safe use of centrifuges, the rotor must be balanced during use (Box 44.1), or the spindle and rotor assembly may be damaged permanently; in severe cases, the rotor may fail and cause a serious accident.

Working safely with centrifuges

Given their speed of rotation and the extremely high forces generated, centrifuges have the potential to be extremely dangerous, if you use them incorrectly.

KEY POINT It is *essential* that you balance your loaded centrifuge tubes before use. As a general rule, balance all sample tubes to within 1% or better, using a top-pan balance or scales. Place balanced tubes opposite each other.

You should only use a particular centrifuge if you fully understand the operating principles: if unsure, check with a member of staff. For safety reasons, all centrifuges are manufactured with an armoured casing that should contain any fragments in cases of rotor failure. Machines usually have a safety lock to prevent the motor from being switched on unless the lid is closed and to stop the lid from being opened while the rotor is moving. Do not be tempted to use older machines without a safety lock, or centrifuges where the locking mechanism is damaged/inoperative. Be particularly careful to make sure that hair and clothing are kept well away from moving parts.

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Bonner, P. and Hargreaves, A. (2011) *Basic Bioscience Laboratory Techniques: A Pocket Guide*. Wiley, New York.

Boyer, R.F. (2011) *Biochemistry Laboratory: Modern Theory and Techniques*, 2nd edn. Benjamin Cummings, San Francisco.

[Also covers other topics, including chromatography and spectroscopy.]

Graham, J.M. (2001) *Biological Centrifugation*. Garland Science, New York.

STUDY EXERCISES

44.1 Decide on the type of centrifuge required for a particular application. What centrifuge would you use for each of the following?

- (a) Separating mitochondria from a cell homogenate.
- (b) Separating yeast cells from their surrounding medium in an experiment to study the uptake of a radiolabelled amino acid as a function of time.
- (c) Harvesting cells from a bioreactor containing 25 L of growth medium.

44.2 Determine centrifugal acceleration for a specific centrifuge. Calculate the centrifugal acceleration (RCF, i.e. the g value) of the following (express your answers to three significant figures):

- (a) A bench centrifuge with an average radius of rotation of 125 mm operating at 4000 r.p.m.
- (b) A bench-top microcentrifuge with an average radius of rotation of 60 mm, operating at 12 000 r.p.m.
- (c) An ultracentrifuge with an average radius of rotation of 186 mm, operating at 30 000 r.p.m.

44.3 Determine the speed required to give a particular centrifugal acceleration (g value). Calculate the speed required (r.p.m. value) for each of the following (express your answers to three significant figures):

- (a) RCF of 1500 g , using a bench centrifuge with an average radius of rotation of 95 mm.
- (b) RCF of 50 000 g , using a high-speed centrifuge with an average radius of rotation of 135 mm.
- (c) RCF of 13 000 g , using a bench-top microcentrifuge with an average radius of rotation of 5.5 cm.

44.4 Calculate the difference in centrifugal acceleration (g value) between the top, middle and bottom of a centrifuge tube. Assuming that the minimum, average and maximum radial distances of a centrifuge tube in a swing-out rotor of a bench centrifuge operating at 5000 r.p.m. are 45 mm, 70 mm and 95 mm respectively, what are the corresponding g values at the top, middle and bottom of the tube when the centrifuge is operating? (Express your answer to three significant figures.)

Answers to these study exercises are available at go.pearson.com/uk/he/resources

45 Measuring light

Table 45.1 Wavelength ranges for UV, visible and IR radiation, compiled from several sources

Type	Wavelength range (nm)
UVC	100–290*
UVB	290–320
UVA	320–400
Violet	390–450
Blue	450–500
Green	500–600
Yellow	560–600
Orange	600–620
Red	620–770
IR	>770

* Note that UVC is absorbed by the Earth's ozone layer.

Light measurement is directly relevant to several aspects of biology, including: photosynthesis, photomorphogenesis and photoperiodism in plants; perception and thermoregulation in animals; and aquatic biology relating to many types of organisms.

Understanding the nature of light

Light is most strictly defined as that part of the spectrum of electromagnetic radiation detected by the human eye. However, the term is also applied to radiation just outside that visible range (for example, UV and infrared 'light'). Electromagnetic radiation is emitted by the sun and by other sources (for example, an incandescent lamp) and the electromagnetic spectrum is a broad band of radiation, ranging from cosmic rays to radio waves (Fig. 45.1). Most biological experiments involve measurements within the UV, visible and infrared regions (generally, within the wavelength range 200–1000 nm, see Table 45.1).

Radiation has the characteristics of a particle and of a vibrating wave, travelling in discrete particulate units, or 'packets', termed photons. A quantum is the amount of energy contained within a single photon (it is important to understand the difference between these two terms, although they are occasionally used interchangeably in the literature). In some circumstances, it is appropriate to measure light in terms of the number of photons, usually expressed directly in moles (6.02×10^{23} photons = 1 mol); older textbooks may use the redundant term Einstein as the unit of measurement, where 1 Einstein = 1 mol photons. Alternatively, the energy content (power) may be measured (for example, in W m^{-2}). Radiation also behaves as a vibrating electrical and magnetic field (wave) moving in a particular direction, with the magnetic and electrical components vibrating perpendicular to one another and perpendicular to the direction of travel.

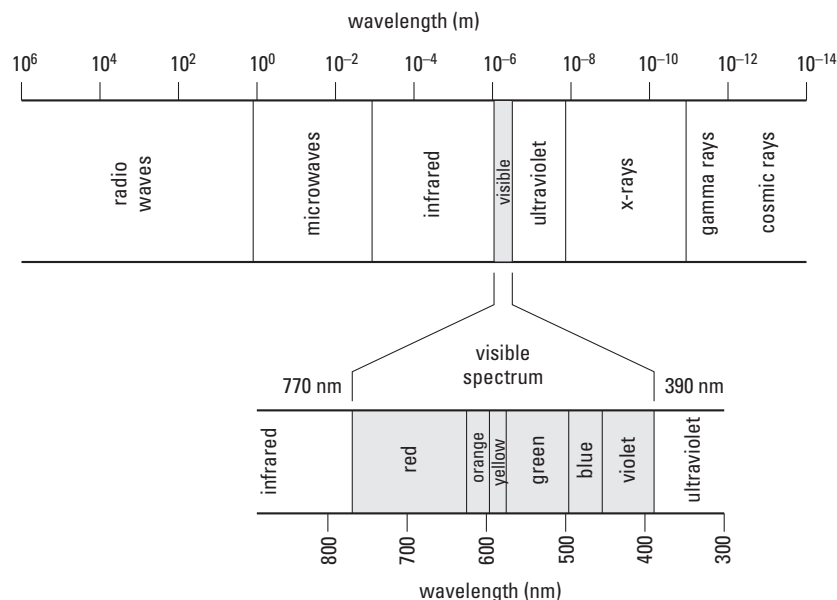


Fig. 45.1 The electromagnetic spectrum.
Methods for Physical and Chemical Analysis of Fresh Waters, 2nd edn, International Biological Programme, No. 8, Blackwell Scientific (Golterman, H.L., Clymo, R.S. and Ohnstad, M.A.M., 1978).

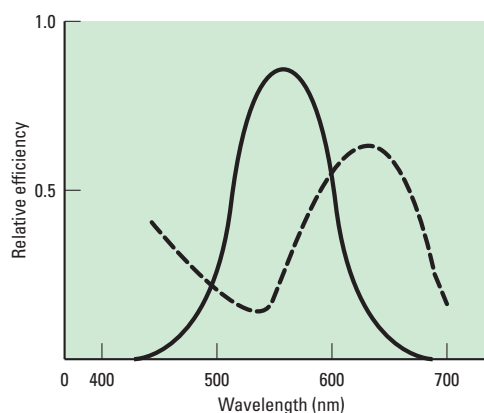


Fig. 45.2 Relative efficiency of vision (solid line) and photosynthesis (dashed line) as a function of wavelength.

The wave nature of radiation gives rise to the concepts of wavelength (λ , usually measured in nm), frequency (ν , measured in s^{-1}), speed (c , the speed of electromagnetic radiation, which is $3 \times 10^8 \text{ m s}^{-1}$ in a vacuum) and direction. In other words, radiation is a vector quantity, where

$$c = \lambda \nu \quad [45.1]$$

Making photometric and radiometric measurements

Photometric measurements

These are based on the energy perceived by a ‘standard’ human eye, with maximum sensitivity in the yellow–green region, around 555 nm (Fig. 45.2). The unit of measurement is the candela, a base unit in the SI system, defined in terms of the visual appearance of a specific quantity of platinum at its freezing point. Derived units are used for the luminous flow (lumen) and luminous flow per unit area (lux). These units were once used in photobiology and you may come across them in older literature. However, it is now recognised that such measurements are of little direct relevance to biologists, including even those who may wish to study visual responses, because they are not based on fundamental physical principles.

KEY POINT The human eye rapidly compensates for changes in light climate by varying the size of the pupil and is a very poor source of information on light quantity. It is important to make light measurements using reliable instruments and to express these measurements in appropriate units.

Radiometric measurements

The radiometric system is based on physical properties of the electromagnetic radiation itself, expressed either as the number of photons, or as their energy content. The following terms are used (units of measurement in parentheses):

- **Photon flux** ($\text{mol photons s}^{-1}$) is the number of photons arriving at an object within the specified time interval.
- **Photon exposure** ($\text{mol photons m}^{-2}$) is the total number of photons received by an object, usually expressed per unit surface area.
- **Photon flux density** ($\text{mol photons m}^{-2} \text{ s}^{-1}$) or PFD is the most commonly used term to describe the number of photons arriving at a particular surface, expressed per unit surface area and per unit time interval.
- **Photosynthetically active radiation** or PAR is radiation within the waveband 400–700 nm, since the photosynthetic pigments (chlorophylls, carotenoids, etc.) show maximum absorption within this band.
- **Photosynthetic photon flux density** ($\text{mol photons m}^{-2} \text{ s}^{-1}$) or PPFD is the number of photons within the waveband 400–700 nm arriving at a particular surface, expressed per unit surface area and per unit time interval. Often this term is used interchangeably with PFD.
- **Irradiance** ($\text{J m}^{-2} \text{ s}^{-1} = \text{W m}^{-2}$) is the amount of energy arriving at a surface, expressed per unit surface area and per unit time interval.
- **Photosynthetic irradiance** (W m^{-2}) or PI is the energy of radiation within the waveband 400–700 nm arriving at a surface, expressed per unit surface area and per unit time interval.

Choosing a measurement scale – this will depend on its relevance to your study. For example, in some environmental studies, you might be interested in total energy input, so irradiance would be a valid measurement; in another type of study, you might be focusing on plant productivity and therefore photosynthetically active radiation, PAR, would be more relevant.

Table 45.2 Approximate conversion factors for a photosynthetic irradiance (PI) of 1 W m^{-2} to photosynthetic photon flux density (PPFD)

Source	PPFD ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)
Sunlight	4.6
'Cool white' fluorescent tube	4.6
Osram 'daylight' fluorescent tube	4.6
Quartz-iodine lamp	5.0
Tungsten bulb	5.0

(Source: Luning, 1981)

Choosing a measurement scale

Photon flux density

This is often the most appropriate unit of measurement for biological systems where individual photons are involved in the underlying process, for example, in photosynthetic studies, where PPFD is measured, since each photochemical reaction involves the absorption of a single photon by a pigment molecule. Most modern light-measuring instruments (radiometers) can measure this quantity, giving a reading in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Irradiance

This is appropriate if you are interested in the energy content of the light, for example, if you are studying energy balance, or thermal effects. Many radiometers measure photosynthetic irradiance within the waveband 400–700 nm, giving a reading in W m^{-2} . It is possible to make an approximate conversion between PPFD and PI measurements, providing the spectral properties of the light source are known (see Table 45.2).

Spectral distribution

This can be determined using a spectroradiometer, for example, to compare different light sources (Fig. 45.3). A spectroradiometer measures irradiance

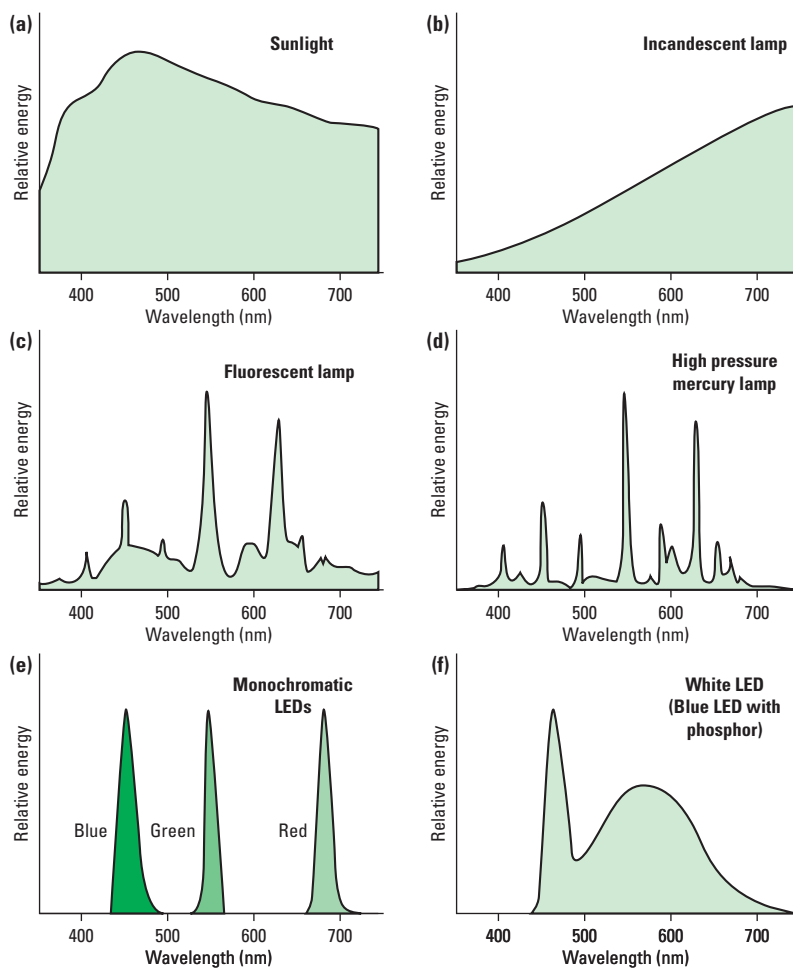


Fig. 45.3 Spectral distribution of energy output from various sources. (Adapted from Gupta and Agarwal, 2017)

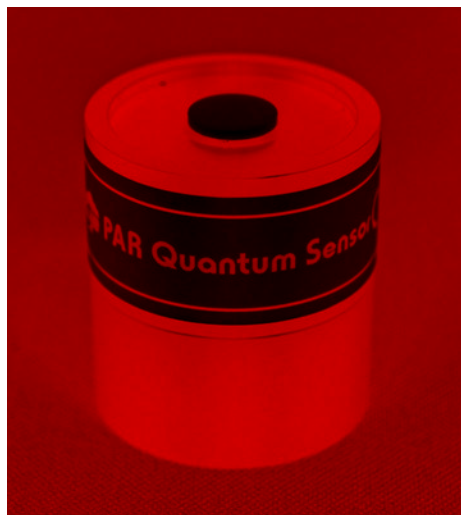


Fig. 45.4 A typical radiometer (quantum) sensor.
Photo courtesy of Skye Instruments Ltd.

or photon flux density in specific wavebands. This instrument consists of a monochromator (p. 339) to allow separate narrow wavebands (5–25 nm bandwidth) to be measured by a detector; some instruments provide a plot of the spectral characteristics of the source.

Measurements can be instantaneous or integrated over a time period (for example, a day). Instantaneous measurements are used in physiological studies to record prevailing conditions while integrated measurements tend to be used in environmental research.

Using a radiometer ('light meter')

The main components of a radiometer are:

- **Receiver (sensor):** either flat-plate, hemispherical or spherical, depending upon requirements. Most incorporate a protective diffuser, to reduce reflection.
- **Detector:** either thermoelectric or photoelectric. Some photoelectric detectors suffer from fatigue, with a decreasing response on prolonged exposure: check the manufacturer's handbook for exposure times.
- **Processor** and readout device to convert the output from the detector into a visible reading, in digital or analogue form.

Figure 45.4 shows a typical flat-plate radiation detector with diffusion plate. Modern instruments may incorporate two sets of receiver–detector assemblies to account for sources of radiation over 360°, and data loggers are capable of presenting instantaneous or integrated readings. Box 45.1 gives practical details of the steps involved in using a radiometer. In your write-up, give full details of how the measurement was made, for example, the type of light source, instrument used, where the sensor was placed, whether an average was calculated, etc.

Box 45.1 How to measure photon flux density or irradiance using a portable radiometer

1. **Check the battery.** Most instruments have a setting that gives a direct readout of battery voltage. Recharge, if necessary, before use.
2. **Select the appropriate type of measurement** (e.g. photon flux density or irradiance over the PAR waveband, or an alternative range): the simpler instruments have a selection dial for this purpose.
3. **Place the sensor in the correct location and position** for the measurement: it may be appropriate to make several measurements at different positions, and take an average.
4. **Choose the most appropriate scale** for the readout device: for needle-type meters, the choice of maximum reading is usually selected by a dial, within the range 0.3 to 30 000. Start at a high range and work down until the reading is on the scale. Your final scale should be chosen to provide the most accurate reading, e.g. a reading of $15 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ should be made using the 0–30 scale, rather than a higher range.
5. **Read the value from the meter.** For needle-type instruments there may be two scales, the upper one marked from 0 to 10 and the lower one from 0 to 3: make sure you use the correct one, e.g. a half-scale deflection on the 0–30 scale is 1.5.
6. **Check that the answer is realistic**, e.g. full sunlight has a PPFD of up to $2000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ($\text{PI} \approx 400 \text{ W m}^{-2}$), though the value will be far lower on a dull or cloudy day, while the PPFD at a distance of 1 m from a mercury lamp is around $150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the same distance from a fluorescent lamp.

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Gupta, S.D. and Agarwal, A. (2017) Artificial lighting system for plant growth and development: chronological advancement, working principles, and comparative assessment. In: *Light Emitting Diodes for Agriculture*, S.D. Gupta (ed.) pp. 1–26. Springer Nature Singapore, Singapore. DOI 10.1007/978-981-10-5807-3_1

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STUDY EXERCISES

45.1 Carry out a calculation involving interconversion of photosynthetic photon flux and photosynthetic irradiance. For sunlight with a photosynthetic photon flux density (PPFD) of 1610 mol m^{-2} , what is the total amount of photosynthetic energy (in joules) falling on a leaf of area 45 cm^2 over a 30-min experimental period?

45.2 Compare the photosynthetic photon flux densities (PPFD) of different locations. The following values represent typical light levels for a range of different situations, expressed either as photosynthetic irradiance (PI) or PPFD:

- (a) outside, on a sunny day: $\text{PI} = 300 \text{ W m}^{-2}$
- (b) in a room lit by 'cool white' fluorescent tubes: $\text{PPFD} = 6.50 \text{ nmol photons cm}^{-2} \text{ s}^{-1}$

- (c) under the leaf canopy in a forest: $\text{PPFD} = 275 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$
- (d) in a growth cabinet lit by a bank of 'daylight' fluorescent lights: $\text{PI} = 35.2 \text{ W m}^{-2}$
- (e) in a room lit by a tungsten bulb: $\text{PI} = 1.15 \text{ mW cm}^{-2}$.

Convert all to PPFD in the same units (expressed to three significant figures) and then rank the locations in order of decreasing PPFD.

45.3 Research the various types of commercial radiometers (light meters) available on the Web. Make a list of the features that you might want to find out about and see how much information you can find.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

46 Carrying out basic spectroscopy and spectrometry

Definitions

Terms used to describe the investigation of interactions between electromagnetic radiation (generally 'light') and matter:

Spectroscopy – the study of the non-destructive interaction (e.g. absorption, emission and scattering of light) between substances and any portion of the electromagnetic spectrum.

Spectrometry – the quantitative investigation of the spectrum of radiation or particle size produced as a result of an interaction (often destructive) between energy and matter, using a spectrometer.

Spectrophotometry – the quantitative investigation of visible, ultraviolet or infrared light emitted, transmitted or reflected as a function of wavelength, using a spectrophotometer.

Photometry – the measurement of light emitted at a specific selected wavelength (or set of discrete wavelengths) following excitation of the sample, quantified using a photometer (note: this term is also used for instruments that measure the intensity of visible or biologically active light).

Definition

Absorbance (A) – this is given by:

$$A = \log_{10}(I_0/I).$$

Usually shown as A_x where 'x' is the wavelength, in nanometres. As an example, for incident light (I_0) = 1.00 and emergent light (I) = 0.16 (expressed in relative terms), $A = \log_{10}(1.00, 0.16) = \log_{10} 6.25 = 0.796$ (to three significant figures).

The absorption and emission of electromagnetic radiation of specific energy (wavelength) are characteristic features of many molecules, involving the movement of electrons between different energy states in accordance with the laws of quantum mechanics. Spectroscopic techniques are used to measure and interpret such interactions between molecules and radiation.

Understanding electromagnetic radiation

Electrons in atoms or molecules are distributed at various energy levels, but are mainly at the lowest energy level, usually termed the ground state. When exposed to energy (for example, from electromagnetic radiation), electrons may be excited to higher energy levels (excited states), with the associated absorption of energy at specific wavelengths giving rise to an absorption spectrum. One quantum of energy is absorbed for a single electron transition from the ground state to an excited state. On the other hand, when an electron returns to its ground state, one quantum of energy is released; this may be dissipated to the surrounding molecules (as heat) or may give rise to an emission spectrum. The energy change (ΔE) for an electron moving between two energy states, E_1 and E_2 , is given by the equation:

$$\Delta E = E_1 - E_2 = h\nu \quad [46.1]$$

where h is the Planck constant (p. 195) and ν is the frequency of the electromagnetic radiation expressed in Hz or s^{-1} . Frequency is related to wavelength (λ , usually expressed in nm) and the speed of electromagnetic radiation, c (p. 327) by the expression:

$$\nu = c \div \lambda \quad [46.2]$$

Electromagnetic radiation in the visible, infra-red and ultraviolet regions of the spectrum is usually referred to as 'light', measured using a spectrophotometer.

Principles of light absorption

Two fundamental principles govern the absorption of light passing through a solution:

1. The absorption of light is exponentially related to the number of molecules of the absorbing solute that are encountered, i.e. the solute concentration $[C]$.
2. The absorption of light is exponentially related to the length of the light path through the absorbing solution, l .

These two principles are combined in the Beer–Lambert relationship (sometimes referred to simply as 'Beer's Law'), which is usually expressed in terms of absorbance (A) – the logarithm of the ratio of the incident light (I_0) to the emergent light (I):

$$A = \epsilon l [C] \quad [46.3]$$

where A is absorbance, ϵ is a constant for the absorbing substance and the wavelength, termed the absorption coefficient or absorptivity, and $[C]$ is expressed either as mol L^{-1} or g L^{-1} and l is given in cm.

Using spectroscopy – this is valuable for:

- tentatively identifying compounds, by determining their absorption or emission spectra
- quantifying substances, either singly or in the presence of other compounds, by measuring the signal strength at an appropriate wavelength
- determining molecular structure
- following reactions, by measuring the disappearance of a substance, or the appearance of a product as a function of time.

Definition

Transmittance (T) – this is usually expressed as a percentage, at a particular wavelength, T_x , where

$$T_x = (I/I_0) \times 100 (\%).$$

As an example, for incident light (I_0) = 1.00 and emergent light (I) = 0.275 (expressed in relative terms) then transmittance, $T = (0.275 \div 1.00) \times 100 = 27.5\%$.

KEY POINT The Beer–Lambert relationship, expressed in mathematical form in eqn [46.3], states that there is a direct linear relationship between the concentration of a substance in a solution, $[C]$, and the absorbance of that solution, A .

This relationship is extremely useful, since most spectrophotometers are constructed to give a direct measurement of absorbance (A), sometimes also termed extinction (E), of a solution (older texts may use the outdated term optical density, OD). Absorbance at a particular wavelength is often shown as a subscript, for example, A_{550} represents the absorbance at 550 nm. The proportion of light passing through the solution is known as the transmittance (T), and is calculated as the ratio of the emergent and incident light intensities.

Some spectrophotometers have two scales:

1. **An exponential scale** from zero to infinity, measuring absorbance.
2. **A linear scale** from 0 to 100, measuring (per cent) transmittance.

For most practical purposes, the Beer–Lambert relationship applies and you should use the absorbance scale.

Carrying out UV/visible spectrophotometry

This is a widely used technique for measuring the absorption of radiation in the visible and UV regions of the spectrum, including the production of absorption spectra. A spectrophotometer is an instrument designed to allow precise measurement at a particular wavelength of radiation, while a colorimeter is a simpler instrument, using filters to measure a broader part of the spectrum, for example, light in the green, red or blue regions of the visible spectrum; Jones *et al.* (2021) provides details of how to use a colorimeter.

The principal components of a UV/visible spectrophotometer are shown in Fig. 46.1. High-intensity tungsten bulbs are used as the light source in basic instruments, capable of operating in the visible region (i.e. 400–700 nm). Deuterium lamps are used for UV spectrophotometry (200–400 nm); these lamps are fitted with quartz envelopes, since glass does not transmit UV radiation.

A major improvement that the spectrophotometer has over the simple colorimeter is the use of a diffraction grating to produce a parallel beam of monochromatic light from the (polychromatic) light source. In practice the light emerging from such a monochromator is not of a single wavelength, but is a narrow band of wavelengths. This bandwidth is an important characteristic, since it determines the wavelengths used in absorption measurements – the bandwidth of basic spectrophotometers is around 5–10 nm, while research instruments have bandwidths of less than 1 nm.

Bandwidth is affected by the width of the exit slit (the slit width), since the bandwidth will be reduced by decreasing the slit width. To obtain accurate data at a particular wavelength setting, the narrowest possible slit width should be used. However, decreasing the slit width also reduces the amount of light reaching the detector, decreasing the signal-to-noise ratio. The extent to which the slit width can be reduced depends upon the sensitivity and stability of the detection/amplification system and the presence of stray light.

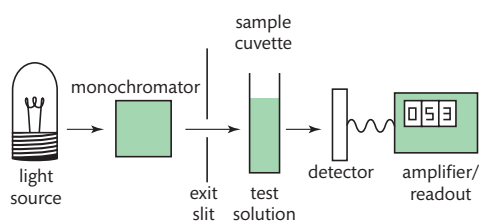


Fig. 46.1 Components of a UV/visible spectrophotometer.

SAFETY NOTE Working with spectrophotometers – take care not to spill aqueous solutions into the inside of the instrument, owing to the risk of electric shock during use (switch off at mains and seek assistance if this should happen).

Using plastic disposable cuvettes – these are adequate for work in the near-UV region, e.g. for enzyme studies using nicotinamide coenzymes, at 340 nm as well as the visible range.

Examples The molar absorptivity of NADH is $6.22 \times 10^3 \text{ L mol}^{-1} \text{ cm}^{-1}$ at 340 nm. For a test solution giving an absorbance of 0.21 in a cuvette with a light path of 5 mm, using eqn [46.3] this is equal to a concentration of:

$$0.21 = 6.22 \times 10^3 \times 0.5 \times [C]$$

$$[C] = 0.000675 \text{ mol L}^{-1}$$

(or $67.5 \mu\text{mol L}^{-1}$).

The specific absorptivity (10 g L^{-1}) of double-stranded DNA is 200 at 260 nm, therefore a solution containing 1 g L^{-1} will have an absorbance of $200/10 = 20$. For a DNA solution, giving an absorbance of 0.35 in a cuvette with a light path of 1.0 cm, using eqn [46.3] this is equal to a concentration of:

$$0.35 = 20 \times 1.0 [C]$$

$$[C] = 0.0175 \text{ g L}^{-1}$$

(equivalent to $17.5 \mu\text{g mL}^{-1}$).

Chlorophylls *a* and *b* of vascular plants and green algae can be extracted in 90% v/v acetone/water, assayed by measuring the absorbance of the mixed solution at two wavelengths, according to the formulae:

$$\text{Chlorophyll } a \text{ (mg L}^{-1}\text{)} = 11.93 A_{664} - 1.93 A_{647}$$

$$\text{Chlorophyll } b \text{ (mg L}^{-1}\text{)} = 20.36 A_{647} - 5.5 A_{664}$$

Note: different equations are required for other solvents.

Most UV/visible spectrophotometers are designed to take cuvettes with an optical path length of 10 mm. Disposable plastic cuvettes are suitable for routine work in the visible range using aqueous and alcohol-based solvents, while glass cuvettes are useful for other organic solvents. Glass cuvettes are manufactured to more exacting standards, so you should use optically matched glass cuvettes for accurate work, especially at low absorbances (< 0.1), where any differences in the optical properties of cuvettes for reference and test samples will be pronounced. Glass and plastic absorb UV light and so cuvettes must be used at wavelengths below 300 nm.

KEY POINT Before taking a measurement, make sure that cuvettes are clean, unscratched, dry on the outside, filled to the correct level and located in the correct position in their sample holders.

Proteins and nucleic acids in biological samples can accumulate on the inside faces of glass/quartz cuvettes, so remove any deposits using acetone on a cotton bud, or soak overnight in 1 mol L^{-1} nitric acid. Corrosive and hazardous solutions must be used in cuvettes with tightly fitting lids, to prevent damage to the instrument and to reduce the risk of accidental spillage.

Basic instruments use photocells similar to those used in simple colorimeters, or photodiode detectors. In many cases, a different photocell must be used at wavelengths above and below 550–600 nm, owing to differences in the sensitivity of such detectors over the visible waveband. The detectors used in more sophisticated instruments give increased sensitivity and stability when compared to photocells.

Digital displays are preferred to traditional needle-type meters, as they are not prone to parallax errors and misreading of the absorbance scale. Some digital instruments can be calibrated to give a direct readout of the concentration of the test substance.

KEY POINT Basic spectrophotometers are most accurate within the absorbance range from 0.00 to 1.00 and your calibration standards and test solutions should be prepared to give readings within this range.

Basic instruments are single beam spectrophotometers in which there is only one light path. Set the instrument to zero absorbance using a blank solution, and then replace with the test solution, to obtain an absorbance reading. An alternative approach is used in double beam spectrophotometers, where the light beam from the monochromator is split into two separate beams, one beam passing through the test solution and the other through a reference blank. You then obtain absorbance readings via an electronic circuit which compares the output from the reference (blank) and sample cuvettes. Double beam spectrophotometry reduces measurement errors caused by fluctuations in output from the light source or changes in the sensitivity of the detection system, since reference and test solutions are measured at the same time (Box 46.1). Recording spectrophotometers are double beam instruments, designed for use with a data recorder, either by recording the difference in absorbance between reference and test solutions across a predetermined waveband to give an absorption spectrum

Box 46.1 How to operate a spectrophotometer

- 1. Switch on and select the correct lamp** for your measurements (e.g. deuterium for UV, tungsten for visible light).
- 2. Allow up to 15 min for the lamp to warm up** and for the instrument to stabilise before use.
- 3. Select the appropriate wavelength:** on older instruments a dial is used to adjust the monochromator, while newer machines have microprocessor-controlled wavelength selection.
- 4. Select the appropriate detector:** some instruments choose the correct detector automatically (on the basis of the specified wavelength), while others have manual selection.
- 5. Choose the correct slit width** (if available): this may be specified in the protocol you are following, or may be chosen on the manufacturer's recommendations.
- 6. Insert appropriate reference blank(s):** single beam instruments use a single cuvette, while double beam instruments use two cuvettes (a matched pair for accurate work). The reference blanks should match the test solution in all respects apart from the substance under test, i.e. they should contain all reagents apart from this substance. *Make sure that the cuvettes are positioned correctly, with their polished (transparent) faces in the light path, and that they are precisely located in the cuvette holder(s).*
- 7. Check/adjust the 0% transmittance:** most instruments have a control that allows you to zero the detector output in the absence of any light (termed 'dark current' correction). Some microprocessor-controlled instruments carry out this step automatically.
- 8. Set the absorbance reading to zero:** usually via a digital readout or a dial.
- 9. Analyse your samples:** replace the appropriate reference blank with a test sample, allow the absorbance reading to stabilise (5–10 s) and read the absorbance value from the meter/readout device. For absorbance readings greater than 1 (i.e. <10% transmittance), the signal-to-noise ratio is too low for accurate results. Your analysis may require a calibration curve or you may be able to use the Beer–Lambert relationship (eqn [46.3]) to determine the concentration of test substance in your samples.
- 10. Check the scale zero at regular intervals** using a reference blank, e.g. after every 10 samples.
- 11. Check the reproducibility of the instrument:** measure the absorbance of a single solution several times during your analysis. It should give the same value.

Problems (and solutions): inaccurate/unstable readings are most often caused by incorrect use of cuvettes, e.g. dirt, fingerprints or test solution on outside of cuvette (wipe the clear faces using a soft tissue before insertion into the cuvette holder and handle only by the opaque faces), condensation (if cold solutions are not allowed to reach room temperature before use), air bubbles (which scatter light and increase the absorbance; tap gently to remove), insufficient solution (causing refraction of light at the meniscus), particulate material in the solution (check for 'cloudiness' in the solution and centrifuge before use, where necessary) or incorrect positioning in light path (locate in correct position).

(Fig. 46.2), or by recording the change in absorbance at a particular wavelength as a function of time (for example, in an enzyme assay, Chapter 62).

Measuring high absorbances in spectrophotometric analysis – if any final solution has an absorbance that is too high to be read with accuracy on your spectrophotometer (i.e. $A > 2$), it is bad practice to dilute the solution so that it can be measured. This dilutes both the sample molecules and the colour reagents to an equal extent. Instead, you should dilute the original sample and reassay.

Carrying out quantitative spectrophotometric analysis

A single (purified) substance in solution can be quantified using the Beer–Lambert relationship (eqn [46.3]), provided its absorptivity is known at a particular wavelength (usually at the absorption maximum for the substance, since this will give the greatest sensitivity). The molar absorptivity is the absorbance given by a solution with a concentration of 1 mol L^{-1} ($= 1 \text{ kmol m}^{-3}$) of the compound in a light path of 1 cm. The appropriate value may be available from tabulated spectral data (for example, Anon, 1963), or it can be determined experimentally by measuring the absorbance of known concentrations of the substance (Box 46.1) and plotting a standard curve (see Chapter 43). This should confirm that the

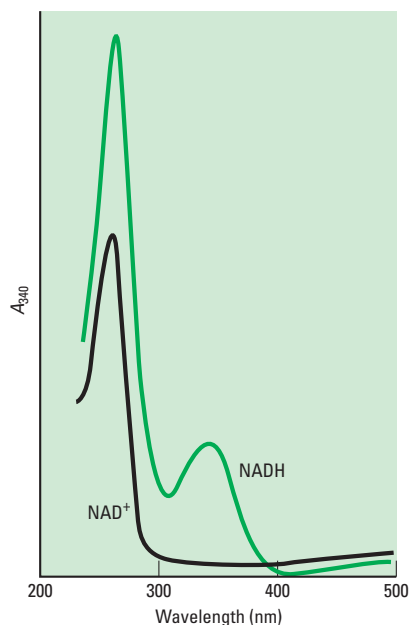


Fig. 46.2 Absorption spectra of nicotinamide adenine dinucleotide in oxidised (NAD^+) and reduced (NADH) form. Note the 340 nm absorption peak (A_{340}), used for quantitative work.

Measuring low absorbances in spectrophotometric analysis – for accurate readings with highly dilute solutions, use a cuvette with a long optical path length (e.g. 5 cm, rather than 1 cm).

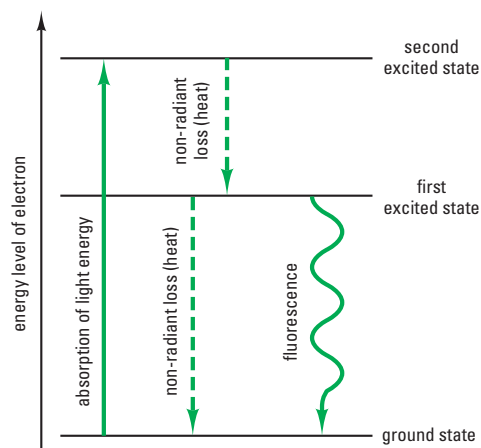


Fig. 46.3 Energy levels and energy transitions in fluorescence.

relationship is linear over the desired concentration range and the slope of the line will give the molar absorptivity.

The specific absorptivity is the absorbance given by a solution containing 10 g L^{-1} (i.e. 1% w/v) of the compound in a light path of 1 cm. This is useful for substances of unknown molecular weight, for example, proteins or nucleic acids, where the amount of substance in solution is expressed in terms of its mass, rather than as a molar concentration. For use in eqn [46.3], the specific absorptivity should be divided by 10 to give the solute concentration in g L^{-1} .

This simple approach cannot be used for mixed samples where several substances have a significant absorption at a particular wavelength. In such cases, it may be possible to estimate the amount of each substance by measuring the absorbance at several wavelengths, for example, protein estimation in the presence of nucleic acids. Further details of spectrophotometric methods of determining the amount of protein in an aqueous sample are given in Chapter 57.

Measuring fluorescence

With most molecules, after electrons are raised to a higher energy level by absorption of electromagnetic radiation, they soon fall back to the ground state by radiationless transfer of energy (heat) to the solvent. However, with some molecules, the events shown in Fig. 46.3 may occur, i.e. electrons may lose only part of their energy by non-radiant routes and the rest may be emitted as electromagnetic radiation, a phenomenon known as fluorescence. Since not all of the energy that was absorbed is emitted (because of non-radiant loss), the wavelength of the fluorescent light is longer than the absorbed light (longer wavelength = lower energy). Thus, a fluorescent molecule has both an absorption spectrum and an emission spectrum. The difference between the excitation wavelength (λ_{ex}) and the emission wavelength (λ_{em}), measured in nm, is known as the Stokes shift, and is fundamental to the sensitivity of fluorescence techniques. The existence of a Stokes shift means that emitted light can be detected against a low background, independently of the excitation wavelength.

Most fluorescent molecules have the following features:

- **a highly conjugated structure** with alternating double and single bonds, involving ring structures, often containing O or N as heteroatoms
- **a condensed system of fused rings**, with one or more heteroatoms
- **electron-donating groups** such as $-\text{OH}$, $-\text{OCH}_3$, $-\text{NH}_2$ and $-\text{NR}_2$, together with electron-attracting groups elsewhere in the molecule, in conjugation with the electron-donating groups
- **a rigid, planar structure**.

Figure 46.4 illustrates many of these features for the molecule fluorescein. This compound is used in many biological applications, including visualisation of nucleic acids (Chapters 25, 26 and 67) and fluorescent antibody tests (Chapter 53). For fluorescein-labelled proteins, linkage to the molecule of interest is created via an isothiocyanate group ($-\text{N}=\text{C}=\text{S}-$) from the carbon labelled * in Fig. 46.4, while for fluorogenic enzyme substrates, linkage to relevant biomolecules is usually via either of the hydroxyl groups. Other examples of organic molecules with intrinsic fluorescence are provided in Table 46.1.

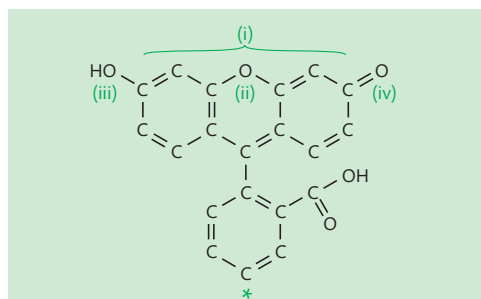


Fig. 46.4 Fluorescein – a widely used fluorescent label, showing (i) a planar conjugated system of fused rings; (ii) heteroatoms within the conjugated structures; (iii) an electron-donating group; and (iv) an electron-attracting group.

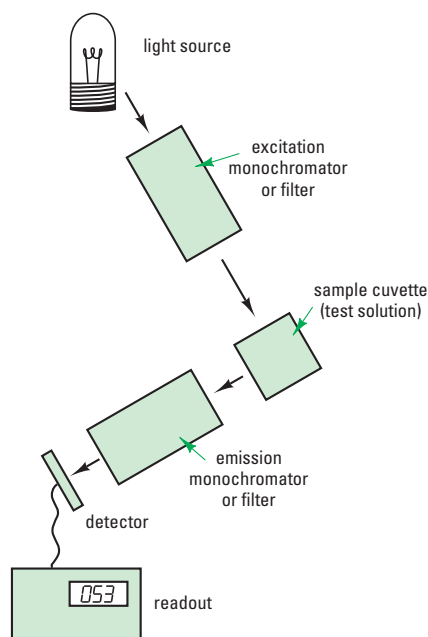


Fig. 46.5 Components of a fluorimeter (fluorescence spectrophotometer). Note that sample cuvettes for fluorimetry must have clear sides all round.

Using fluorogenic enzyme substrates – this is discussed further in Chapter 62.

Using fluorimetry

The principal components of a fluorescence spectrophotometer (fluorimeter) are shown in Fig. 46.5. The instrument contains two monochromators, one to select the excitation wavelength and the other to monitor the light emitted, usually at 90° to the incident beam (though light is actually emitted in all directions). As an example, the wavelengths used to measure the highly fluorescent compound aminomethylcoumarin are 388 nm (excitation) and 440 nm (emission).

Compared with UV/visible spectrophotometry, fluorescence spectroscopy has certain advantages, including the following:

- **Enhanced sensitivity** (up to 1000-fold), since the emitted light is detected against a background of zero, in contrast to spectrophotometry where small changes in signal are measured against a large ‘background’ (see eqn [46.3]).
- **Increased specificity**, because two specific wavelengths are used in the process, one to excite and one for emission. However, there are also certain drawbacks:
- **Not all compounds show intrinsic fluorescence**, limiting its application. However, some non-fluorescent compounds may be coupled to fluorescent dyes, or fluorophores (for example, proteins may be coupled to fluorescamine).
- **The light emitted can be less than expected due to quenching**, i.e. when substances in the sample (for example, oxygen) either interfere with energy transfer, or absorb the emitted light (in some instances, the sample molecules may self-quench if they are present at high concentration).

The sensitivity of fluorescence has made it invaluable in techniques in which specific antibodies are linked to a fluorescent dye, including:

- **fluorescence immunoassay (FIA)**
- **immunohistochemistry**, which requires the use of a fluorescence microscope, for example, using fluorescent antibodies, or fluorescent *in situ* hybridisation (FISH) for nucleic acid detection
- **flow cytometry** – a fluorescence-activated cell sorter (FACS) uses cell surface protein-specific monoclonal antibodies labelled with fluorescent dyes to separate and enumerate cells such as lymphocytes.

Measuring phosphorescence and luminescence

A phenomenon related to fluorescence is phosphorescence, which is the emission of light following intersystem crossing between electron orbitals (for example, between excited singlet and triplet states). Light emission in phosphorescence usually continues after the exciting energy is no longer applied and, since more energy is lost in intersystem crossing, the emission wavelengths are generally longer than with fluorescence. Phosphorescence has limited applications in biomolecular sciences.

Luminescence (or chemiluminescence) is another phenomenon in which light is emitted, but here the energy for the initial excitation of electrons is provided by a chemical reaction rather than by electromagnetic radiation.

Table 46.1 Examples of compounds with intrinsic fluorescence. Chlorophyll fluorescence in particular is discussed further in Chapter 64.

Pigments

Chlorophyll, Phycobilins (e.g. phycocyanin and phycoerythrin)

Vitamins

Riboflavin, vitamins A, B6, and E, nicotinamide

Drugs

acetylsalicylic acid (Asprin), morphine, barbiturates, propranolol, ampicillin, tetracyclines

Pollutants

Naphthalene, anthracene, benzo[a]pyrene

SAFETY NOTE Working in atomic spectroscopy – the use of high-pressure gas cylinders can be particularly hazardous. Always consult a member of staff before using such apparatus.

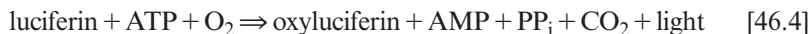
SAFETY NOTE Carrying out acid digestion – always work within a fume hood and wear gloves and safety glasses throughout the procedure. Rinse any spillages with a large volume of water.

Plotting calibration curves in quantitative analysis – do not force your calibration line to pass through zero if it clearly does not. There is no reason to assume that the zero value is any more accurate than any other reading you have made.

Choosing atomic absorption spectrophotometry over flame photometry – reasons include:

- improved sensitivity
- increased precision
- decreased interference.

An example is the action of the enzyme luciferase, extracted from fireflies, which catalyses the following reaction:



The light produced is either yellow–green (560 nm) or red (620 nm). This system can be used in biomolecular analysis of ATP, either to determine ATP concentration in a biological sample, or to follow a coupled reaction. Measurement can be performed using the photomultiplier tubes of a scintillation counter (pp. 405–6) to detect the emitted light, with calibration of the output using a series of standards of known ATP content.

Carrying out atomic spectrophotometry

Atoms of certain metals will absorb and emit radiation of specific wavelengths when heated in a flame, in direct proportion to the number of atoms present. Atomic spectrophotometric techniques measure the absorption or emission of particular wavelengths of UV and visible light, to identify and quantify such metals.

Using flame atomic emission spectrophotometry (flame photometry)

The principal components of a flame photometer are shown in Fig. 46.6. A liquid sample is converted into an aerosol in a nebuliser (atomiser) before being introduced into the flame, where a small proportion (typically fewer than 1 in 10 000) of the atoms will be raised to a higher energy level, releasing this energy as light of a specific wavelength, which is passed through a filter to a photocell detector. Flame photometry is used to measure the alkali metal ions K^+ , Na^+ and Ca^{2+} in biological fluids; Box 46.2 gives details of the basic procedure.

Using atomic absorption spectrophotometry (flame absorption spectrophotometry)

This technique is applicable to a broad range of metal ions, including those of Pb, Cu, Zn, etc. It relies on the absorption of light of a specific wavelength by atoms dispersed in a flame. The appropriate wavelength is provided by a cathode lamp, coated with the element to be analysed, focused through the flame and onto the detector. When the sample is introduced into the flame, it will decrease the light detected in direct proportion to the amount of metal present. Newer variants of this method include flameless atomic absorption spectrophotometry and atomic fluorescence spectrophotometry, both of which are more sensitive than the flame absorption technique.

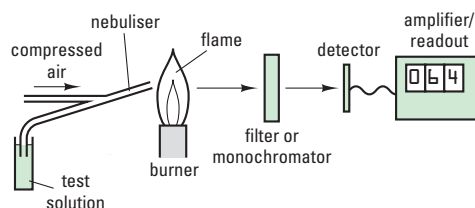


Fig. 46.6 Components of a flame photometer.

Box 46.2 How to operate a flame photometer

When using a flame photometer:

- **Allow time for the instrument to stabilise.** Switch on the instrument, light the flame and wait at least 5 min before analysing your solutions.
- **Check for impurities in your reagents.** For example, if you are measuring K^+ in an acid digest of some biological material, check the K^+ content of a reagent blank containing everything except the biological material, processed in exactly the same way as the samples. Subtract this value from your sample values to obtain the true K^+ content. If using acid digestion, always work within a fume hood and wear gloves and safety glasses at all times. Rinse any spillages with a large volume of water.
- **Quantify your samples using a calibration curve (Chapter 43).** Calibration standards should cover the expected concentration range for the test solutions – your calibration curve may be non-linear (especially at concentrations above 1 mmol L^{-1} , i.e. 1 mol m^{-3} in SI units).
- **Analyse all solutions in duplicate**, so that reproducibility can be assessed.
- **Check your calibration.** Make repeated measurements of a standard solution of known concentration after every six or seven samples, to confirm that the instrument calibration is still valid.
- **Consider the possibility of interference.** Other metal atoms may emit light which is detected by the photocell, since the filters cover a wider waveband than the emission line of a particular element. This can be a serious problem if you are trying to measure low concentrations of a particular metal in the presence of high concentrations of other metals (e.g. Na^+ in sea water), or other substances that form complexes with the test metal, suppressing the signal (e.g. phosphate). Atomic absorption spectrophotometry is an alternative approach for such samples.

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STUDY EXERCISES

46.1 Write a protocol for using a spectrophotometer.

After reading this chapter, prepare a detailed stepwise protocol explaining how to use one of the spectrophotometers in your department. Ask another student or a tutor to evaluate your protocol and provide you with feedback.

46.2 Use the Beer–Lambert relationship in quantitative spectrophotometric analysis. Calculate the following (express your answer to three significant figures):

- The concentration of NADH ($\mu\text{mol L}^{-1}$) in a test solution giving an absorbance at 340 nm (A_{340}) of 0.53 in a cuvette with a path length of 1 cm, based on a molar absorptivity for NADH of $6220 \text{ L mol}^{-1} \text{ cm}^{-1}$ at this wavelength.
- The amount of NADH (nmol) in 20 mL of a test solution where $A_{340} = 0.62$ in a cuvette with a path length of 5 cm, based on a molar absorptivity for NADH of $6220 \text{ L mol}^{-1} \text{ cm}^{-1}$ at this wavelength.
- The mass concentration ($\mu\text{g mL}^{-1}$) of double-stranded DNA in a test solution giving an absorbance at 260 nm (A_{260}) of 0.57 in a cuvette of path length 5 mm, based on an absorptivity of $20 \text{ L g}^{-1} \text{ cm}^{-1}$.
- The amount (ng) of double-stranded DNA in a 50 μL subsample from a test solution where $A_{260} = 0.31$ in a cuvette of path length 1 cm, based on an absorptivity of $20 \text{ L g}^{-1} \text{ cm}^{-1}$.
- The chlorophyll a content, in $\mu\text{g (g fresh weight)}^{-1}$, of a plant leaf of fresh weight 1.56 g extracted in 50 mL of 90% acetone, where $A_{664} = 0.182$ at 664 nm and $A_{646} = 0.035$, based on the following equation:

$$\text{Chlorophyll a (mg L}^{-1}\text{)} = 11.93 A_{664} - 1.93 A_{646}.$$

46.3 Determine the molar absorptivity of a substance in aqueous solution. A solution of *p*-nitrophenol containing $8.8 \mu\text{g mL}^{-1}$ gave an absorbance of 0.535

at 404 nm in a cuvette of path length 1 cm. What is the molar absorptivity of *p*-nitrophenol at 404 nm, expressed to three significant figures? (Note: M_r of *p*-nitrophenol is 291.27.)

46.4 Determine the concentration of metal ions based on atomic spectroscopy of test and standard solutions. The following data represent a set of calibration standards for K^+ in aqueous solution, measured by flame photometry:

Absorbance of standard solutions containing K^+ at up to 0.5 mmol L^{-1}

K^+ concentration (mmol L^{-1})	Absorbance
0	0.000
0.1	0.155
0.2	0.279
0.3	0.391
0.4	0.537
0.5	0.683

Draw a calibration curve using the above data and use this to estimate the amount of K^+ in a test sample prepared by digestion of 0.482 g of tissue in a final volume of 25 mL of solution, giving an absorbance of 0.429 when measured at the same time as the standards shown above. Express your answer in $\mu\text{mol K}^+ (\text{g tissue})^{-1}$, to three significant figures. See also study exercise 42.1 for a similar exercise, based on atomic absorption spectrophotometry of Zn.

46.5 Explain the basis of fluorescence. Why is the wavelength of the emitted light longer than that used to excite a fluorescent molecule?**46.6 Account for the difference in design between spectrophotometer and fluorimeter cuvettes.** Why does a spectrophotometer cuvette have only two clear sides while a fluorimeter cuvette has four clear sides?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

47 Using advanced spectroscopy and spectrometry

Identifying compounds – the techniques described in this chapter can often provide sufficient information to identify a compound with a low probability of error. Chapter 56 gives further detail on identification and analysis of biomolecules.

While Chapter 46 deals with spectroscopy and spectrometry in the UV/visible parts of the electromagnetic spectrum, this chapter focusses on methods which analyse radiation in other regions (Table 47.1), and includes techniques which rely on the analysis of responses when molecules are subjected to magnetic fields or the detection of molecular fragments when molecules are bombarded with energy. The instruments used in these methods are extremely sensitive and require detailed technical knowledge to operate, so relevant techniques and results may only be discussed and/or demonstrated in the early stages of your course. For instance, having covered relevant theory, you might be given spectra to interpret and analyse. The various types of spectroscopy, and their relationship with the electromagnetic spectrum, are shown in Table 47.1.

Using infrared (IR) and Raman spectroscopy

Both of these techniques involve the measurement of frequencies produced by the vibration of chemical bonds (bending and stretching). The IR/Raman region of the electromagnetic spectrum is generally considered to be from 800 to 2500 nm (for near-IR) and up to 16 000 nm (for mid-IR). Near-IR spectroscopy involves recording the spectrum in that region in a manner analogous to UV/visible spectroscopy (Chapter 46), and quantitative analysis is possible. However, the most widely used technique is mid-IR spectroscopy, which allows identification of groups or atoms in a sample compound, but is inappropriate for quantitative measurement. A peak at a particular frequency can be identified by reference to libraries or computer databases of IR spectra, for example a peak at a wavenumber of $1730\text{--}1750\text{ cm}^{-1}$ corresponds to a carbonyl group ($\sim\text{C=O}$), which is present in fatty acids and proteins. The $1400\text{--}600\text{ cm}^{-1}$ region is often described as the ‘fingerprint’ region because no two compounds give identical spectra.

Understanding the origins of IR and Raman spectra – the IR spectrum is caused by changes in charge displacement in bonds. The Raman spectrum (named after physicist C.V. Raman) is caused by changes in polarisability in bonds.

The value of IR spectroscopy is greatly enhanced by Fourier transformation (FT), named after the mathematician J.B. Fourier. FT is a procedure for interconverting frequency functions and time or distance functions. In FT-IR, information is obtained from an interferometer, which splits the incident beam so that it passes through both the sample and a reference. When the beam is recombined, interference patterns arise because the two path lengths are different. The interference pattern has the same relationship to a normal spectrum as a hologram has to a picture, and

Table 47.1 The electromagnetic spectrum and types of spectroscopy. Techniques for the UV/visible region are discussed in Chapter 46.

Type of radiation	Origin	Wavelength	Type of spectroscopy
γ -rays	Atomic nuclei	$<0.1\text{ nm}$	γ -ray spectroscopy
X-rays	Inner shell electrons	$0.1\text{--}1.0\text{ nm}$	X-ray fluorescence (XRF)
Ultraviolet (UV)	Ionisation	$10\text{--}200\text{ nm}$	UV spectroscopy
UV/visible	Valency electrons	$200\text{--}800\text{ nm}$	UV/visible spectroscopy
Infrared	Molecular vibrations	$0.8\text{--}25\text{ }\mu\text{m}$	Infrared spectroscopy (IR) and Raman
Microwaves	Electron spin alignment	$400\text{ }\mu\text{m--}30\text{ cm}$	Electron spin resonance (ESR)
Radiowaves	Nuclear spin resonance	$>100\text{ cm}$	Nuclear magnetic resonance (NMR)

integral computers use FT to convert the pattern into a spectrum in under a minute. The overall result is a greatly enhanced signal-to-noise ratio.

Understanding IR spectra

A typical IR spectrum is shown in Fig. 47.1 and you should note the following points:

- **The x axis, the wavelength of the radiation, is given in wavenumbers ($\bar{\nu}$), the reciprocal of the wavelength, expressed in reciprocal centimetres (cm^{-1}).** Wavenumber is a term used widely in IR spectroscopy, but rarely in other types of analysis. You may still see some spectra from old instruments using microns (μ , equivalent to the SI unit ‘micrometres’, μm , at 1×10^{-6} m) for wavelength.
- **The y axis, expressing the amount of radiation absorbed by the molecule, is usually shown as % transmittance.** When no radiation is absorbed (all is transmitted through the sample) there is 100% transmittance, while 0% transmittance implies that all radiation is absorbed at a particular wavenumber. Since the y axis scale goes from 0 to 100% transmittance, the absorption peaks are displayed *downwards* from the 100% line; this is in contrast to most other common spectra.
- **The cells holding the sample usually display imperfections and are not completely transparent to IR radiation, even when empty.** Therefore, the base line of the spectrum is rarely set on 100% transmittance and quantitative applications of IR spectroscopy are more complex than for UV/visible spectrophotometry (p. 339).

Using wavenumber in IR spectroscopy – this is a well-established convention, since high wavenumber = high frequency = high energy = short wavelength. Expression of the IR range, 4000 cm^{-1} to 650 cm^{-1} , is in ‘easy’ numbers and the high energy is found on the left-hand side of the spectrum. Note that IR spectroscopists often refer to wavenumbers as ‘frequencies’, e.g. ‘the peak of the $\text{C}=\text{O}$ stretching “frequency” is at 1720 cm^{-1} ’.

Using IR to study metabolism – the rapid analysis possible with gaseous samples makes IR ideal for studying CO_2 metabolism in photosynthesis and respiration.

Applications of IR and Raman spectroscopy

The principal use of IR and Raman spectroscopy is in the identification of drugs (for example penicillin), small peptides, pollutants and food contaminants. When an IR spectrometer is coupled to a gas chromatograph (p. 350), it can be used for the analysis of drug metabolites.

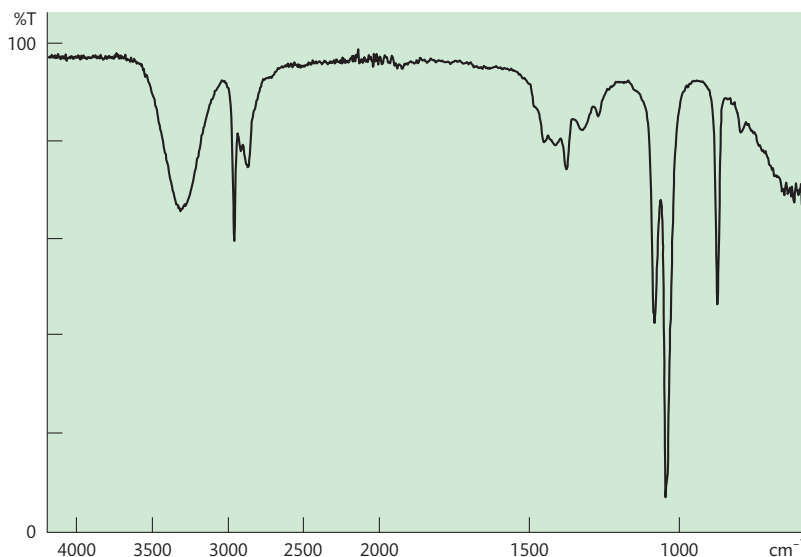


Fig. 47.1 IR spectrum for ethanol.

SAFETY NOTE Working with NMR spectrometers – these instruments generate very high magnetic fields and exert strong magnetic forces on materials such as steel items brought close to the magnet (<1 m). NMR magnets can also destroy data stored in magnetic form – keep items such as memory sticks, laptops and credit cards well away.

Example For an external magnetic field of 2.5 T (Tesla), ΔE for ^1H is $6.6 \times 10^{-26}\text{J}$, and, since $\Delta E = h\nu$, the corresponding frequency (ν) is 100 MHz; for ^{13}C in the same field, ΔE is $1.7 \times 10^{-26}\text{J}$, and ν is 25 MHz.

One possible future use of IR is for the non-invasive analysis of organic compounds that are of diagnostic significance (for example glucose in diabetes). This involves illuminating a small area of skin with IR radiation and using the resultant IR spectrum to quantify a specific compound: an example of such a system is the Diamontech non-invasive blood glucose monitor (<https://www.diamontech.de/home>).

Using nuclear magnetic resonance (NMR)

Electromagnetic signals (at radiofrequencies of 1–500 MHz) are produced by atomic nuclei in response to magnetic fields and can be used to identify and monitor compounds. This is possible because of differences in the magnetic states of atomic nuclei, involving very small transitions in energy levels. The atomic nuclei of isotopes of many elements are magnetic because they are charged and have spin. The magnetic nuclei most often studied in biology are ^1H , ^{13}C , ^{14}N , ^{15}N , ^{19}F and ^{31}P . When these nuclei interact with a uniform external magnetic field, they behave like tiny compass needles and align themselves in a direction either parallel or antiparallel to the field. The two orientations have different energies, with the parallel direction having a lower energy than the antiparallel (Fig. 55.3). The energy difference between the two levels (ΔE) corresponds to a precise electromagnetic frequency (ν), according to similar quantum principles to those for the excitation of electrons (p. 326). When a sample containing an isotope with a magnetic nucleus is placed in a magnetic field and exposed to an appropriate radiofrequency, transitions between the energy levels of magnetic nuclei will occur when the energy gap and the applied frequency are in *resonance* (i.e. when they are matched exactly). Differences in energy levels, and hence resonance frequencies (ν_0), depend on the magnitude of the applied magnetic field (B_0) and the magnetogyric ratio (λ), according to the equation:

$$\nu_0 = \lambda B_0 / 2\pi \quad [47.1]$$

The magnetogyric ratio varies from one isotope to another, so NMR is performed at different frequencies for different nuclei at any given value of B_0 . The principal components of an NMR spectrometer are shown in Fig. 55.4.

For magnetic nuclei in a given molecule, an NMR spectrum is generated because, in the presence of the applied field, different nuclei experience different local magnetic fields depending on the arrangement of electrons in their vicinity. The effective field (B) at the nucleus can be expressed as:

$$B = B_0(1 - \sigma) \quad [47.2]$$

where σ (the shielding constant) expresses the contribution of the small secondary field generated by nearby electrons. The magnitude of σ is dependent on the electronic environment of a nucleus, so nuclei in different environments give rise to different resonance frequencies, according to the equation:

$$\nu_0 = \lambda B_0(1 - \sigma) / 2\pi \quad [47.3]$$

Measuring chemical shifts – ‘ppm’ is not a concentration term in NMR, but is used to reflect the very small frequency changes that occur relative to the reference standard, measured in proportional terms.

The separation of resonance frequencies from a reference value is termed the *chemical shift*, and is expressed in dimensionless terms, as parts per million (ppm). By convention, the chemical shift is positive if the sample nucleus is less shielded than the reference and negative if it is more shielded.

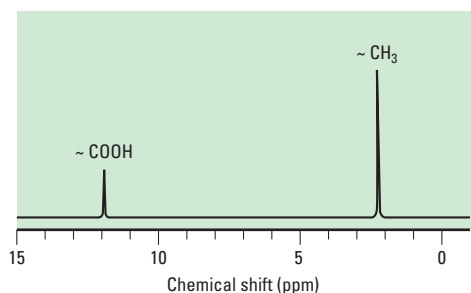


Fig. 47.2 ^1H NMR spectrum of acetic acid (CH_3COOH). The relative areas of the two signals are 1 : 3 and the frequencies (chemical shifts) are expressed in terms of ppm, relative to the reference signal (tetramethyl silane, TMS).

Carrying out NMR using living tissues – reactions can be studied non-destructively, in ‘real time’.

Understanding NMR spectra of biomolecules

Figure 47.2 illustrates the principle underlying this technique – the different chemical environments of the hydrogen atoms in a simple biomolecule such as acetic acid result in two different ^1H resonances or ‘signals’, one corresponding to protons in $\sim\text{CH}_3$, and the other from protons in $\sim\text{COOH}$. Furthermore, the relative intensities of the NMR signals, as measured by their areas, are proportional to the number of contributing nuclei, so the relative areas of the peaks due to the protons in $\sim\text{CH}_3$ and (undissociated) $\sim\text{COOH}$ would be 3:1. Similarly, with ^{31}P NMR of a biologically important molecule such as ATP, there are signals corresponding to the α , β and γ phosphates, the P nuclei of which are in different chemical environments (Fig. 47.3). Thus every molecule that contains one or more magnetic nuclei has its own characteristic NMR fingerprint that may be used for identification and analysis. Spectra such as those shown in Figs 47.2 and 47.3 can be obtained using FT of a large number of individual responses to radiowave pulses. Other factors such as the spin-lattice relaxation time (Jacobson, 2007) can affect signal intensities (peak sizes), and resonances may be split into several lines owing spin–spin coupling (interactions between neighbouring nuclei).

In terms of resolution, narrow signals are obtained only from molecules that are fairly mobile, so most high-resolution NMR studies are carried out using solutions. Since many metabolites in biological samples (cells, tissues, etc.) are in aqueous solution and freely mobile, they can give rise to high-resolution spectra, as shown in Fig. 47.3 for the ^{31}P -containing compounds, ATP, phosphocreatine and inorganic phosphate. On the other hand, ^{31}P signals from less mobile molecules such as DNA or phospholipids are very broad, and can be as wide as the whole scale shown in Fig. 47.3.

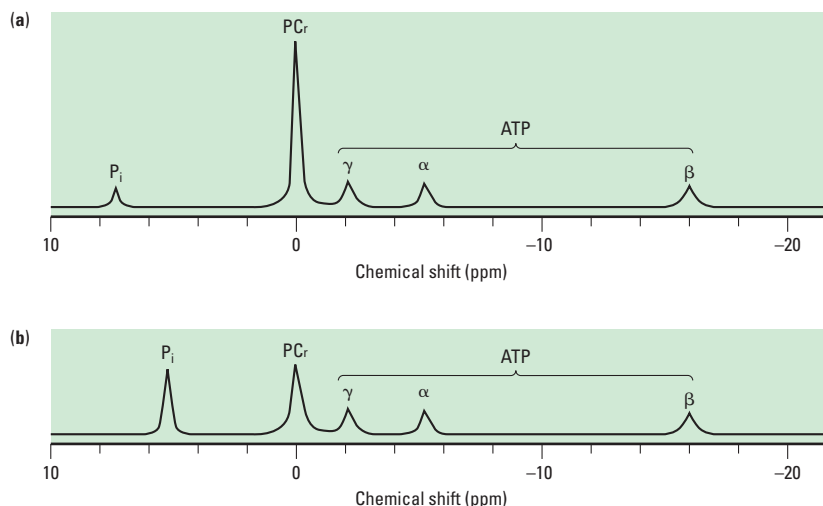


Fig. 47.3 Typical ^{31}P NMR spectrum from intact skeletal muscle (a) at rest and (b) after a fatiguing series of contractions. In (b) ATP levels are preserved at the expense of phosphocreatine (PCr). Also, hydrolysis of ATP during contraction results in a large phosphate (P_i) peak which is shifted to the right compared to (a), reflecting the decrease in intracellular pH that accompanies glycolysis.

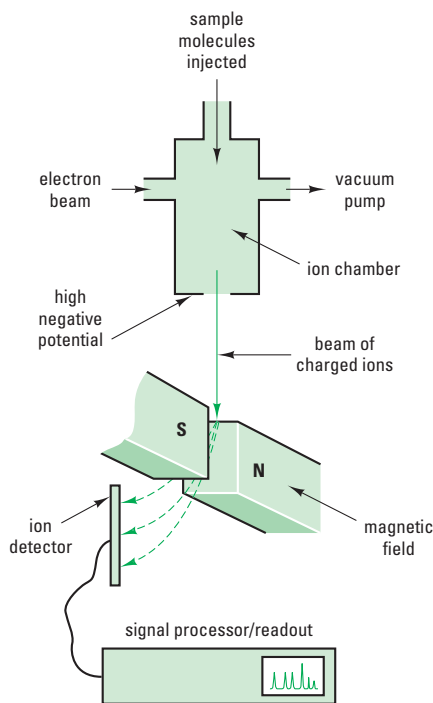


Fig. 47.4 Components of an electron-impact mass spectrometer.

Example IRMS is used in the diagnosis of gastric ulcers caused by *Helicobacter pylori*. This bacterium produces the enzyme urease, degrading urea to CO_2 and NH_3 . A patient drinks a solution of ^{13}C -labelled urea and, if the organism is present in the stomach, $^{13}\text{CO}_2$ will appear at up to 5% v/v in the patient's breath, compared with the expected natural abundance value of 1.1% v/v.

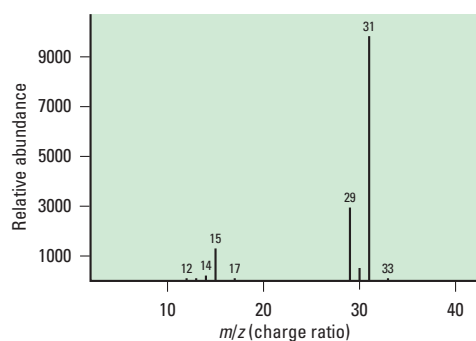


Fig. 47.5 Mass spectrum for methanol.

Using electron spin resonance (ESR)

This technique is based on the energy transitions of spinning electrons in a magnetic field. As with NMR, the low energy state occurs when the electromagnetic field generated by the spinning electron is parallel to the externally generated field, while the high energy state occurs when the electron-generated field is antiparallel. ESR is very useful for studying metalloproteins and can be used to monitor the activity of such proteins (for example cytochrome oxidase) in intact mitochondria or chloroplasts. It can also be used to detect free radicals, for example in irradiated foodstuffs.

Using mass spectrometry (MS)

This technique involves the disintegration of organic compounds into fragment ions in a gas phase. These ions are accelerated to specific velocities using an electric field and then separated on the basis of their different masses. Each fragment of a particular mass is detected sequentially with time. The most widely used method in MS is electron impact ionisation (EI), where the electron source is a heated metal, such as a tungsten filament, subjected to an appropriate potential gradient. The stream of emitted electrons may then interact with a biomolecule (M) in the sample by either:

- 1. electron removal** – where an electron in a bond within the sample molecule is displaced by bombarding electrons, leaving the bond with an unpaired electron and resulting in the production of a cationic free radical, i.e. $\text{M} + \text{e}^- \rightarrow \text{M}^+ + 2\text{e}^-$;
- 2. electron capture** – where addition of an extra electron results in the production of an anionic free radical, i.e. $\text{M} + \text{e}^- \rightarrow \text{M}^\bullet$.

Where it is not known whether cations or anions are formed, the radical is given the symbol \cdot , i.e. as M^\cdot . The first ions formed (parent ions) are unstable, and rapidly undergo further disintegration to give smaller fragments and daughter ions, subsequently separated in the mass spectrometer (Fig. 47.4), to give a mass spectrum such as that shown in Fig. 47.5.

For *in vivo* metabolic studies, the technique of isotope ratio mass spectroscopy (IRMS) is useful since it removes the need to use radioactive isotopes. This technique exploits the ability of MS to distinguish between isotopes such as ^{13}C and ^{12}C . For example, compounds containing ^{13}C have a greater mass than the same compound containing ^{12}C and can be differentiated in the mass spectrum. By selectively labelling a key metabolite with a non-radioactive isotope, the fate of this metabolite can be followed by MS analysis of sequential samples. Normally, the materials used are combusted in oxygen to give gases such as $^{13}\text{CO}_2$ and $^{15}\text{NO}_2$, followed by exposure of such gases to an EI source; useful metabolic data can be obtained by determining the isotope ratio, for example of $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$.

Pyrolysis-mass spectrometry (PY-MS)

This is another variant of mass spectroscopy that is useful for biomolecular applications. The molecules in a sample are decomposed and volatilised (pyrolysed) by heating to a precisely controlled high temperature for a specific time period. The volatile material is then removed by a vacuum, ionised by EI and subjected to MS. Complex mixtures of pyrolysis

Example A useful application of PY-MS is in microbial identification, especially with small samples of organisms that are difficult to culture, e.g. mycobacteria.

products are produced, but interpretation is made easier by computer-based multivariate analysis.

Fast atom bombardment–mass spectrometry (FAB-MS)

This technique is particularly useful for biomolecular applications because it can be used with aqueous solutions. The solution containing the analyte is mixed with glycerol and applied to a probe which is inserted into a vacuum chamber. The mixture is bombarded with a high velocity stream of atoms (usually argon or xenon) rather than electrons, which induces fragmentation of the biomolecules and allows production of the mass spectrum. One problem with FAB-MS is the ‘suppression effect’ phenomenon – with a mixed sample, not all components may be equally accessible to the atomic bombardment depending on the way they are distributed in the sample–glycerol mixture. This can be overcome by coupling FAB-MS to techniques that can give high resolution separation of components, for example gas chromatography (GC, p. 350), high-performance liquid chromatography (HPLC, p. 349), or capillary electrophoresis (pp. 376–7). Greaves and Roboz (2013) give further information on biomolecular aspects of MS.

Text references

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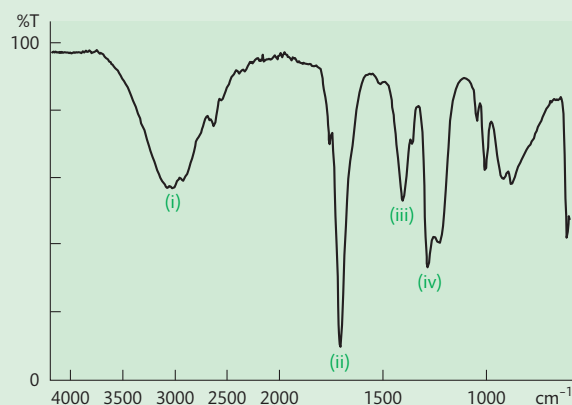
Keeler, J. (2010) *Understanding NMR Spectroscopy*, 2nd edn. Wiley, New York.

STUDY EXERCISES

47.1 Interpret IR signals. The figure shows the IR spectra of acetic acid (ethanoic acid). Using the information provided below, identify the resonance corresponding to (a) the O—H and (b) C=O of the carboxylic acid group in the acetic acid spectrum.

Typical IR absorption ranges for different functional groups

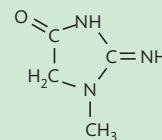
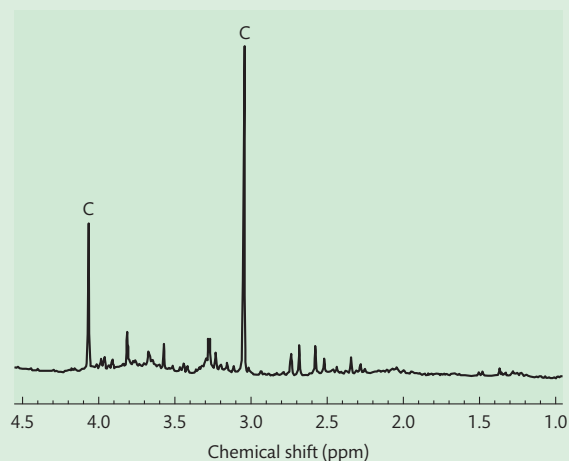
Bond	Location	Wavenumber (cm ⁻¹)
C—O	esters, alcohols	1000–1300
C=O	ketones, aldehydes, carboxylic acids, esters, amides	1680–1750
O—H	carboxylic acids (H-bonded)	2500–3300
O—H	alcohols (H-bonded)	3230–3350
O—H	free	3580–3670
N—H	amines	3100–3500



47.2 Investigate appropriate reference compounds for biological applications of NMR. Why are reference compounds important when measuring chemical shifts in NMR? Which compounds are suitable references for (a) ¹H, (b) ¹³C and (c) ³¹P NMR?

47.3 Test your understanding of the NMR terminology. Distinguish between the term ppm as used in NMR and as a concentration term (p. 163).

47.4 Interpret NMR signals from biomolecules in body fluids. A ¹H NMR spectrum (range 1–4.5 ppm) of a human urine sample obtained from a healthy adult is shown in the first figure below. The spectrum reveals a large number of components/peaks. The two most intense of these peaks (at approximately 3.05 and 4.05 ppm) correspond to creatinine (C), which is abundant in healthy urine. The molecular structure of creatinine is shown in the second figure below. Explain why the ¹H NMR 'fingerprint' of creatinine in urine consists of just two discrete peaks.



Answers to these study exercises are available at go.pearson.com/uk/he/resources

48 Separating compounds using chromatography

Making compromises in chromatography – the process is often a three-way compromise between:

- separation of analytes
- time of analysis
- volume of eluent.

Thus, if you have a large sample volume and you want to achieve a good separation of a mixture of analytes, the time taken for chromatography will be lengthy.

SAFETY NOTE The solvents used as the mobile phases of chromatographic systems are often toxic and may produce noxious fumes – where necessary, work in a fume hood.

Chromatography is used to separate the individual constituents within a sample on the basis of differences in their physical characteristics, for example, molecular size, shape, charge, volatility, solubility and/or adsorptivity. The essential components of a chromatographic system are as follows:

- **a stationary phase:** either a solid, a gel or an immobilised liquid, held by a support matrix
- **a chromatographic bed:** the stationary phase may be packed into a glass or metal column, spread as a thin layer on a sheet of glass or plastic, or adsorbed on cellulose fibres (paper)
- **a mobile phase:** either a liquid or a gas which acts as a solvent, carrying the sample through the stationary phase and eluting from the chromatographic bed
- **a delivery system:** to pass the mobile phase through the chromatographic bed
- **a detection system:** to monitor the test substances (Chapter 49).

Individual substances interact with the stationary phase to different extents as they are carried through the system, enabling separation to be achieved. The more complex chromatographic systems are computer-controlled and require technical knowledge to set up and operate. They may also take time to run. Consequently, you are most likely to use more simple set-ups in the lab and/or to use more advanced methods under close guidance.

KEY POINT In a chromatographic system, those substances which interact strongly with the stationary phase will be retarded to the greatest extent while those which show little interaction will pass through with minimal delay, leading to differences in distances travelled or elution times.

Performing chromatography

Chromatographic systems can be categorised according to the form of the chromatographic bed, the nature of the mobile and stationary phases and the method of separation.

Thin-layer chromatography (TLC) and paper chromatography

Here, you apply the sample as a single spot near one end of the sheet or plate (a glass or plastic support with the stationary phase as a thin attached layer), using a microsyringe or microcapillary. Allow the sheet to dry fully, then transfer to a glass tank containing a shallow layer of solvent (Fig. 48.1). Remove the sheet when the solvent front has travelled across 80–90% of its length.

You can express movement of an individual substance in terms of its relative frontal mobility, or R_F value, where:

$$R_F = \frac{\text{distance moved by substance}}{\text{distance moved by solvent}} \quad [48.1]$$

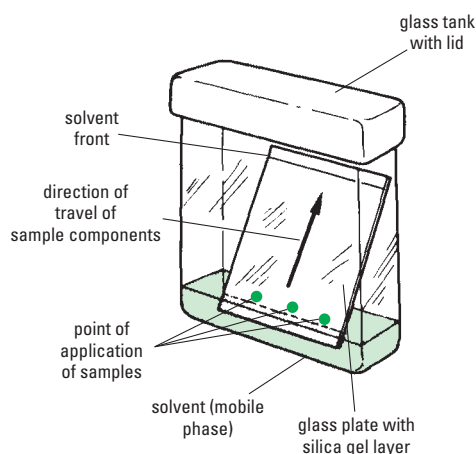


Fig. 48.1 Components of a TLC system.

Using a TLC system – it is essential that you allow the solvent to pre-equilibrate in the chromatography tank for at least 2 h before use, to saturate the atmosphere with vapour. Deliver drops of sample with a blunt-ended microsyringe. Make sure you know exactly where each sample is applied, so that R_F values can be calculated.

Employing 2-Dimensional TLC – in this process, after one elution, you rotate the plate by 90°, and then use a different solvent for a second elution.

Understanding silica gel codes – silica gel is manufactured as particles of 10–15 μm diameter, containing pores of diameter 40, 60, 80, 100 or 150 Å (Ångstrom, p. 194), where $\text{Å} = 10^{-10} \text{ m}$ (thus, silica gel 60 has a pore size of 60 Å). Silica gel G contains calcium sulfate to assist binding to the glass support, while silica gel H has no binder.

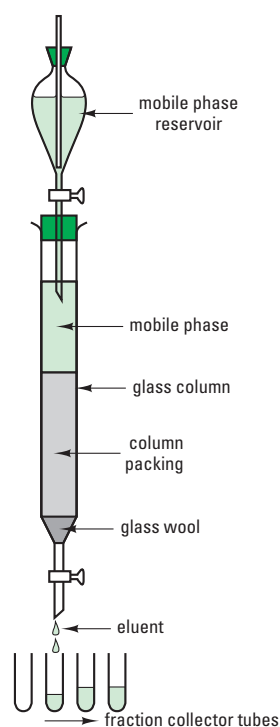


Fig. 48.2 Equipment for column chromatography (gravity feed system).

Alternatively, you can express movement with respect to a standard of known mobility, as R_X , where:

$$R_X = \frac{\text{distance moved by test substance}}{\text{distance moved by standard}} \quad [48.2]$$

The R_F (or R_X) value is a constant for a particular substance and solvent system (under standard conditions) and closely reflects the partitioning of the substance between the stationary and mobile phases. Tabulated values are available for a range of biological molecules and solvents (for example, Stahl, 1969, or Touchstone, 1992). However, you should analyse one or more reference compounds on the same sheet as your unknown sample, to check their R_F values.

Silica gel TLC plates are available commercially, and different grades of gel in the stationary phase can be obtained for specific purposes. After separation, different regions of gel can be scraped from the glass to allow extraction and further analysis of compounds.

Column chromatography

Here, you pack a glass column with the appropriate stationary phase and equilibrate the mobile phase by passage through the column, either by gravity (Fig. 48.2), or using a low-pressure peristaltic pump. You can then introduce the sample to the top of the column, to form a discrete band of material. This is then flushed through the column by the mobile phase. If the individual substances have different rates of migration, they will separate within the column, eluting at different times as the mobile phase travels through the column.

You can detect eluted substances by collecting the mobile phase as it elutes from the column in a series of tubes (discontinuous monitoring), either manually or with an automatic fraction collector. Fractions of 2–5% of the bed volume are usually collected and analysed, for example, by chemical assay. You can now construct an elution profile (or chromatogram) by plotting the amount of substance against either time, elution volume or fraction number, which should give a symmetrical peak for each substance (Fig. 48.3).

You can express the migration of a particular substance at a given flow rate in terms of its retention time (t), or elution volume (V_e). The separation efficiency of a column is measured by its ability to distinguish between two similar substances, assessed in terms of:

- **selectivity** (α), measured using the following equation, which takes into account the retention times of the two peaks (i.e. t_a and t_b), plus the column dead time (t_0):

$$\alpha = \frac{t_b - t_0}{t_a - t_0} \quad [48.3]$$

The column dead time is the time it takes for an unretained compound to pass through the column without any interaction with the stationary phase.

- **resolution** (R), quantified in terms of the retention time and the base width (W) of each peak:

$$R = \frac{2(t_a - t_b)}{W_a + W_b} \quad [48.4]$$

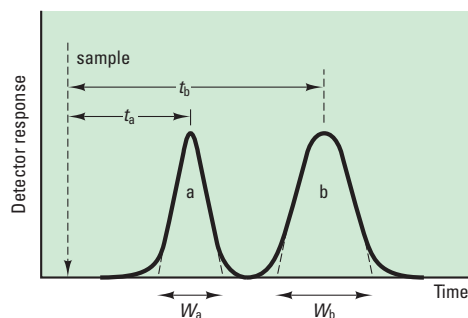


Fig. 48.3 Peak characteristics in a chromatographic separation, i.e. a chromatogram. For symbols, see eqn [48.4].

where the subscripts a and b refer to substances a and b, respectively (Fig. 48.3). For most practical purposes, R values of 1 or more are satisfactory, corresponding to 98% peak separation for symmetrical peaks.

Non-symmetrical peaks may result from column overloading, co-elution of solutes, poor packing of the stationary phase or interactions between the substances and the support material.

As well as columns that you set up for yourself, manufactured columns are also available. For example, single-use silica ‘spin columns’ are used for the chromatographic purification of nucleic acids, with the aid of centrifugation. Here, you add a sample to the top of the column, where the nucleic acids bind to the stationary phase. Then, you rinse with buffer, to remove other constituents. Finally, you elute the nucleic acids using deionized water or elution buffer. Rinsing and elution are facilitated by microcentrifugation (p. 321), since the spin column is designed to fit inside a 1.5 mL microcentrifuge tube.

High-performance liquid chromatography (HPLC)

Column chromatography originally used large ‘soft’ stationary phases that required low-pressure flow of the mobile phase to avoid compression; separations were usually time-consuming and of low resolution (‘low performance’). Subsequently, the production of small, incompressible, homogeneous particulate support materials and high-pressure pumps with reliable, steady flow rates have enabled high-performance systems to be developed. These systems operate at pressures up to 10 MPa, forcing the mobile phase through the column at a high flow rate to give rapid separation with reduced band broadening, owing to smaller particle size.

HPLC columns are usually made of stainless steel, and all components, valves, etc., are manufactured from materials that can withstand the high pressures involved. The two main systems are:

- **isocratic separation:** a single solvent (or solvent mixture) is used throughout the analysis
- **gradient elution separation:** the composition of the mobile phase is altered using a microprocessor-controlled gradient programmer, which mixes appropriate amounts of two different substances to produce the required gradient.

Most HPLC systems are linked to a continuous monitoring detector of high sensitivity, for example, you can detect proteins may spectrophotometrically by monitoring the absorbance of the eluent at 280 nm as it passes through a flow cell (cuvette). Other detectors can be used to measure changes in fluorescence, current or potential (Chapter 49). Most detection systems are non-destructive, which means that you can collect eluent with an automatic fraction collector for further study (Fig. 48.4).

The speed and sensitivity of HPLC make this the method of choice for the separation of many small molecules of biological interest, normally using reverse-phase partition chromatography (p. 351). Separation of macromolecules (especially proteins and nucleic acids) usually requires ‘biocompatible’ systems in which stainless steel components are replaced by titanium, glass or fluoroplastics, using lower pressures to avoid denaturation, for example, the Pharmacia FPLC system. You can carry out such separations using ion-exchange, gel permeation and/or hydrophobic interaction chromatography (pp. 351–3).

Using HPLC – this is a versatile form of chromatography, used with a wide variety of stationary and mobile phases, to separate individual compounds of a particular class of molecules on the basis of size, polarity, solubility or adsorption characteristics.

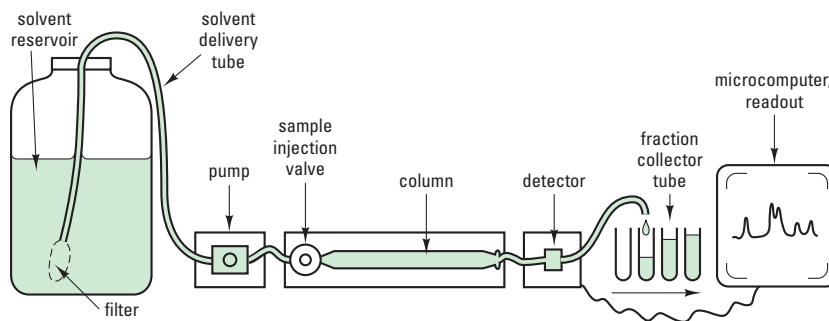


Fig. 48.4 Components of an HPLC system.

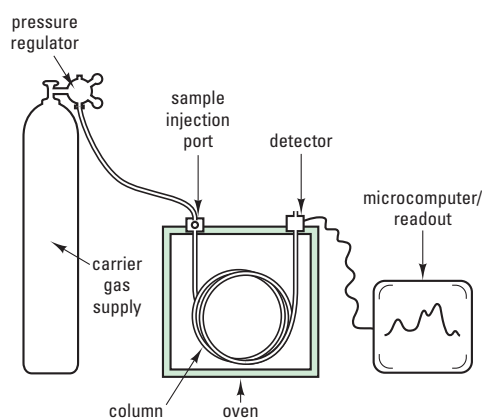


Fig. 48.5 Components of a GC system.

Applying gas chromatography – GC is used to separate volatile, non-polar compounds: substances with polar groups must be converted to less polar derivatives prior to analysis, in order to prevent adsorption on the column, resulting in poor resolution and peak tailing.

Gas chromatography (GC)

Modern GC uses capillary chromatography columns (internal diameter 0.1–0.5 mm) up to 50 m in length (Fig. 48.5). The stationary phase is generally a cross-linked silicone polymer, coated as a thin film on the inner wall of the capillary: at normal operating temperatures, this behaves in a similar manner to a liquid film but is far more robust. The mobile phase ('carrier gas') is usually nitrogen or helium. You can achieve selective separation as a result of the differential partitioning of individual compounds between the carrier gas and silicone polymer phases. The separation of most biomolecules is influenced by the temperature of the column, which may be constant during the analysis ('isothermal' – usually 50–250 °C) or, more commonly, may increase in a pre-programmed manner (for example, from 50 °C to 250 °C at 10 °C per min). You inject samples onto the 'top' of the column, through a sample injection port containing a gas-tight septum. The output from the column can be monitored by flame ionisation, electron capture or thermal conductivity (Chapter 49).

Spectrometric detection systems include mass spectrometry (GC-MS) and infrared spectroscopy (GC-IR). GC can only be used with samples capable of volatilisation at the operating temperature of the column, for example, short chain fatty acids. Other substances may need to be chemically modified to produce more volatile compounds, for example, long chain saturated fatty acids (Chapter 58) are usually analysed as methyl esters while monosaccharides (Chapter 59) are converted to their trimethylsilyl derivatives.

Using chromatography to separate sample constituents

Often, chromatographic systems are used as part of the preliminary purification process for biological samples, followed by further processing and investigation of eluted fractions (for example, as part of DNA analysis).

Adsorption chromatography

This is a form of solid–liquid chromatography. The stationary phase is a porous, finely divided solid which adsorbs molecules of the test substance on its surface owing to dipole–dipole interactions, hydrogen bonding and/ or van der Waals interactions (Fig. 48.6). The range of adsorbents is limited, for example, polystyrene-based resins (for non-polar molecules), silica, aluminium oxide and calcium phosphate (for polar molecules).

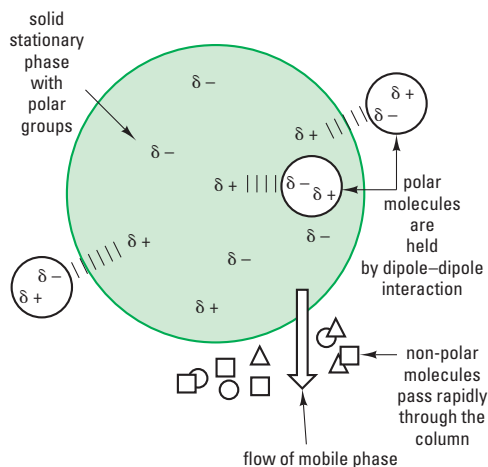


Fig. 48.6 Adsorption chromatography (polar stationary phase).

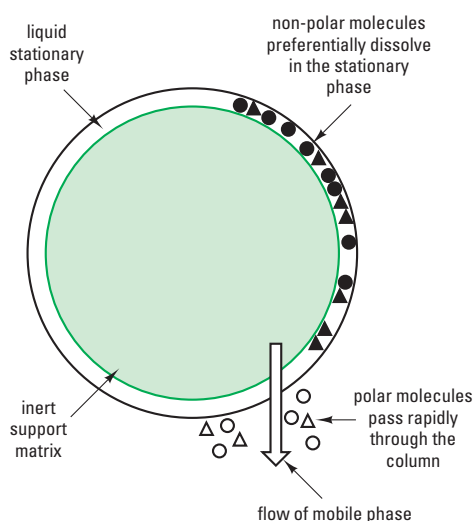


Fig. 48.7 Liquid-liquid partition chromatography, e.g. reverse-phase HPLC.

Selecting a separation method – it is often best to select a technique that involves direct interaction between the substance(s) and the stationary phase (e.g. ion exchange, affinity or hydrophobic interaction chromatography), owing to their increased capacity and resolution compared to other methods (e.g. partition or gel permeation chromatography).

You must activate most adsorbents by heating to 110–120°C before use, since their adsorptive capacity is significantly decreased in the presence of bound water. You can carry out adsorption chromatography in column or thin-layer form, using a wide range of organic solvents.

Partition chromatography

This is based on the partitioning of a substance between two liquid phases, in this instance the stationary and mobile phases. Substances that are more soluble in the mobile phase will pass rapidly through the system while those which favour the stationary phase will be retarded (Fig. 48.7). In normal phase partition chromatography the stationary phase is a polar solvent, usually water, supported by a solid matrix (for example, cellulose fibres in paper chromatography) and the mobile phase is an immiscible, non-polar organic solvent. For reverse-phase partition chromatography the stationary phase is a non-polar solvent (for example, a C18-hydrocarbon, such as octadecylsilane) which is chemically bonded to a porous support matrix (for example, silica), while the mobile phase can be chosen from a wide range of polar solvents, usually water or an aqueous buffered solution containing one or more organic solvents, for example, acetonitrile. Solutes interact with the stationary phase through non-polar interactions and so the *least* polar solutes elute last from the column. You can control solute retention and separation by changing the composition of the mobile phase (for example, % v/v acetonitrile). Reverse-phase high-performance liquid chromatography (RP-HPLC) is used to separate a broad range of non-polar, polar and ionic biomolecules, including peptides, proteins, oligosaccharides and vitamins.

Ion-exchange chromatography (IEC)

Here, separations are carried out using a column packed with a porous matrix which has a large number of ionised groups on its surfaces, i.e. the stationary phase is an ion-exchange resin. The groups may be cation or anion exchangers, depending upon their affinity for positive or negative ions. The net charge on a particular resin depends on the pK_a of the ionisable groups and the pH of the solution, in accordance with the Henderson–Hasselbalch equation (p. 167).

For most practical applications, you should select the ion-exchange resin and buffer pH so that the test substances are strongly bound by electrostatic attraction to the ion-exchange resin on passage through the system, while the other components of the sample are rapidly eluted (Fig. 48.8). You can then elute the bound components by raising the salt concentration of the mobile phase, either step-wise or as a continuous gradient, so that exchange of ions of the same charge occurs at oppositely charged sites on the stationary phase. Weakly bound sample molecules will elute first, while more strongly bound molecules will elute at a higher concentration. Computer-controlled gradient formers are available: if two or more components cannot be resolved using a linear salt gradient, an adapted gradient can be used in which the rate of change in salt concentration is decreased over the range where these components are expected to elute. To maximise resolution in IEC (and HIC) keep your columns as short as possible. Once the sample components have been separated, you should elute them as quickly as possible from the column to avoid band broadening resulting from diffusion of sample ions in the mobile phase.

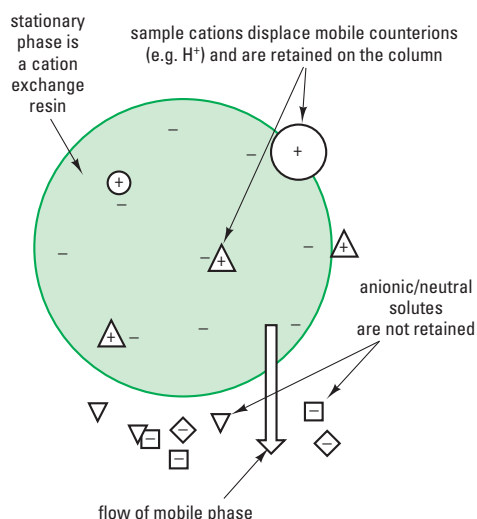


Fig. 48.8 Ion-exchange chromatography (cation exchanger).

Using a gel permeation system – keep your sample volume as small as possible, to minimise band broadening due to dilution of the sample during passage through the column.

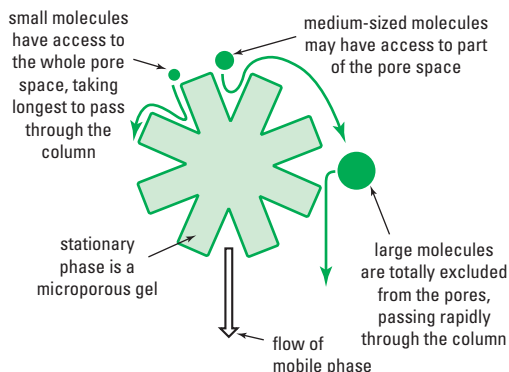


Fig. 48.9 Gel permeation chromatography

You can use IEC to separate mixtures of a wide range of ionic biomolecules, including amino acids, peptides, proteins and nucleotides. Electrophoresis (Chapters 50 and 51) is an alternative means of separating charged biomolecules.

Gel permeation chromatography (GPC; molecular-exclusion chromatography) or gel filtration

Here, the stationary phase is in the form of beads of a cross-linked gel containing pores of a discrete size (Fig. 48.9). The size of the pores is controlled so that at the molecular level, the pores act as ‘gates’ that will exclude large molecules and admit smaller ones. However, this gating effect is not an all or nothing phenomenon: molecules of intermediate size partly enter the pores. A column packed with such beads will have within it two effective volumes that are potentially available to sample molecules in the mobile phase, i.e. V_i , the volume surrounding the beads, and V_{ii} , the volume within the pores. If you place a sample at the top of such a column, the mobile phase will carry the sample components down the column, but at different rates according to their molecular size. A very large molecule will have access to all of V_i but to none of V_{ii} , and will therefore elute in the minimum possible volume (the ‘void volume’, or V_o , equivalent to V_i). A very small molecule will have access to all of V_i and all of V_{ii} , and therefore it has to pass through the total liquid volume of the column (V_t , equivalent to $V_i + V_{ii}$) before it emerges. Molecules of intermediate size have access to all of V_i but only part of V_{ii} , and will elute at a volume between V_o and V_t , in order of decreasing size depending on their access to V_{ii} .

Cross-linked dextrans (for example, Sephadex), agarose (for example, Sepharose) and Polyacrylamide (for example, Bio-gel) can be used to separate mixtures of macromolecules, particularly enzymes, antibodies and other globular proteins (see Table 48.1 for details). Selectivity in GPC is solely dependent on the stationary phase, with the mobile phase being used solely to transport the sample components through the column. A plot of elution volume (V_e) against \log_{10} molecular mass is approximately linear, so, you can estimate the molecular mass of a sample component by calibrating a given column using molecules of known molecular mass and similar shape. A further application of GPC is the general separation of low molecular mass and high molecular mass components, for example, desalting a protein extract by passage through a Sephadex G-25 column is faster and more efficient than dialysis.

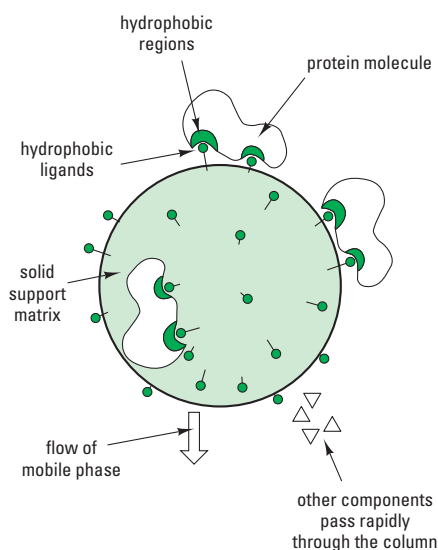
Hydrophobic interaction chromatography (HIC)

This technique allows you to separate proteins, and exploits the fact that many proteins have hydrophobic sites, with hydrophobic amino acid residues (for example, leucine, isoleucine, valine, phenylalanine) on their surfaces. Proteins differ in the nature and extent of these hydrophobic regions. The underlying principle is similar to that of RP-HPLC in that it involves hydrophobic interactions between the sample components and a non-polar stationary phase. However, RP-HPLC is only useful for analytical separations of proteins where retention of biological activity is not required, and is unsuitable for separation of native proteins for several reasons:

- the stationary phase in RP-HPLC columns tends to be densely packed with hydrophobic groups, leading to tight protein binding, possibly through multi-site attachment

Table 48.1 Fractionation ranges of selected gel permeation chromatography media

M_r	Medium
50–1000	Sephadex G15 Bio-gel P-2
1000–5000	Sephadex G-25
1500–30 000	Sephadex G-50 Bio-gel P-10
4000–150 000	Sephadex G-100
5000–250 000	Sephadex G-200
20 000–1 500 000	Sephacryl S 300
60 000–20 000 000	Sepharose 4B

**Fig. 48.10** Hydrophobic interaction chromatography. Hydrophobic interactions between ligands and hydrophobic amino acid residues release 'structured' water, making the interactions thermodynamically favourable.

Using HIC for purification – HIC can be used immediately after salt precipitation, when the salt concentration is high. If the desired biomolecule is then eluted using a gradient of reducing salt concentration, it may be possible to follow HIC directly by IEC (which requires an initial buffer of low ionic strength) without changing the buffer.

- the use of polar organic solvents may lead to protein denaturation
- the high pressures used to obtain rapid flow rates in HPLC may also denature proteins.

The groups used on HIC stationary phases are both less densely packed and less hydrophobic than those used in RP-HPLC, and this results in milder adsorption of proteins (octyl or phenyl groups are commonly used in HIC, rather than octadecyl groups). Furthermore, retention and elution can be achieved using aqueous solutions so that an individual protein can be isolated with its 3D conformation intact.

Separations are based on interactions between the three components of the system, i.e. the hydrophobic stationary phase, the hydrophobic sample molecules and the aqueous stationary phase. In an aqueous environment, hydrophobic groups tend to associate, and this results in certain proteins binding to the stationary phase, where the strength of binding is related to the degree of hydrophobicity of the protein (Fig. 48.10). This tendency is promoted by the presence of certain salts, most commonly ammonium sulfate, that produce 'salting out' effects. You can elute components by a variety of means, for example, by:

- reducing the ammonium sulfate concentration of the mobile phase, to decrease the 'salting out' effect
- changing the salt in the mobile phase to one that does not promote salting out
- including non-ionic detergents (for example, Triton X-100) to reduce hydrophobic interactions
- including aliphatic alcohols, reducing the polarity of the mobile phase; changing the pH or
- reducing the temperature.

Affinity chromatography (AC)

Affinity chromatography enables you to purify biomolecules on the basis of their biological specificity rather than by differences in physicochemical properties, and a high degree of purification (>1000-fold) can be expected. You will find it especially useful for isolating small quantities of material from large amounts of contaminating substances. The technique involves the immobilisation of a complementary binding substance (the ligand) onto a solid matrix in such a way that the specific binding affinity of the ligand is preserved. When a biological sample is applied to a column packed with this affinity support matrix, the molecule of interest will bind specifically to the ligand, while contaminating substances will be washed through with buffer (Fig. 48.11). You can elute the desired molecule by changing the pH or ionic strength of the buffer, to weaken the non-covalent interactions between the molecule and the ligand, or by the addition of other substances that have greater affinity for the ligand.

In AC, the ligand must show specific, but reversible, interaction with the molecule to be purified. It must also contain a reactive functional group, independent of the biospecific site, that will allow covalent attachment to the matrix. The support matrix must be free of non-specific adsorption effects and have sufficient reactive functional groups for the attachment of ligands. Agarose is an ideal support matrix for use in AC. If the ligand is small and the molecule to be purified is large, binding may be restricted

Avoiding protein precipitation in

HIC – certain protein components may precipitate in high ionic strength buffers. If this occurs, dilute your sample and inject larger volumes.

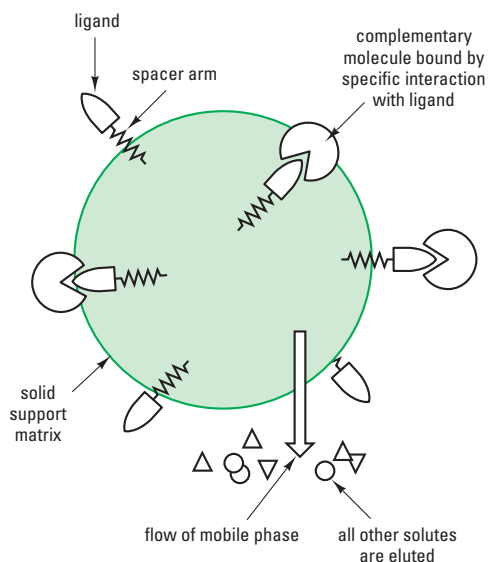


Fig. 48.11 Affinity chromatography.

Examples Biospecific molecules used in affinity chromatography include:

- enzymes and inhibitors/cofactors/substrates;
- hormones and receptor proteins;
- antibodies and antigens;
- complementary base sequences in DNA and RNA.

Eluting substances from an affinity

system – make sure that your elution conditions do not affect the interaction between the ligand and the stationary phase, or you may elute the ligand from the column.

owing to the proximity of the matrix surface. This problem may be overcome by the introduction of a ‘spacer arm’ (for example, a hexane group) between the ligand and the matrix (Fig. 48.11).

A potential disadvantage of the specificity of AC is that you may need to seek a new ligand for each individual separation – a potentially time-consuming and expensive process. It may be more practical to use ‘group-specific adsorbents’ which contain ligands that have an affinity for a class of biochemically related substances. Examples of group-specific adsorbents include the following:

- **Lectins**, which are a group of proteins produced from fungi, plants and animals, that bind reversibly with specific sugar residues. They are very useful for the purification of sugar-containing macromolecules such as glycoproteins, serum lipoproteins and membrane proteins such as receptors. Different lectins show different specificities, for example, concanavalin A and lentil lectin bind to sugars having —OH groups at C-3, C-4 and C-5 (i.e. mannose and glucose), while wheatgerm lectin binds to *N*-acetyl glucosamine residues. Once bound, substances can be resolved by eluting the column with a salt gradient, or by including free sugars to act as competitive binding agents. Lectins are sometimes called agglutinins because of their ability to agglutinate different types of erythrocyte (for example, A, B and O) by binding to the specific receptors on their surfaces.
- **Protein A**, which is a commercially available surface protein from the bacterium *Staphylococcus aureus* that has a specific binding capacity for the constant (F_c) region of IgG-type antibodies from most mammalian species (Fig. 48.12). As well as being useful in the purification of IgG, protein A can also be used for the isolation of other molecules, as long as an antibody can be raised against them. This is possible because interaction of protein A with IgG occurs via the F_c segment of immunoglobulins of this class, irrespective of their F_{ab} components (see Fig. 43.1). Immobilisation of a specific IgG antibody onto a protein A-agarose column produces an affinity support that can be used to purify the desired antigen.
- **Immobilised dyes**, which can be used to purify a wide range of enzymes and proteins. One of the most widely used dyes is Cibacron Blue F3G-A, available as an affinity support as Blue Sepharose. This dye has some structural similarities to nucleotide cofactors such as NAD^+ and $NADP^+$, and is useful for the purification of enzymes that require such cofactors. Chemical modification of such dyes increases their specificity, and it is likely that computer modelling will play a role in the future development of new dye-based affinity supports. Dyes are also used in the related non-chromatographic technique of affinity precipitation. This makes use of two dye molecules linked via a spacer molecule; when this ‘bis-ligand’ is added to a solution containing the molecule to be purified, the ligand specifically binds two desired molecules, forming an insoluble complex. Alternatively, a heterobifunctional ligand can be used – a combination of a specific ligand and a group that can be used to initiate precipitation once the desired molecule has bound to the ligand. Such interactions are equivalent to the precipitin reaction in immunology (p. 393).

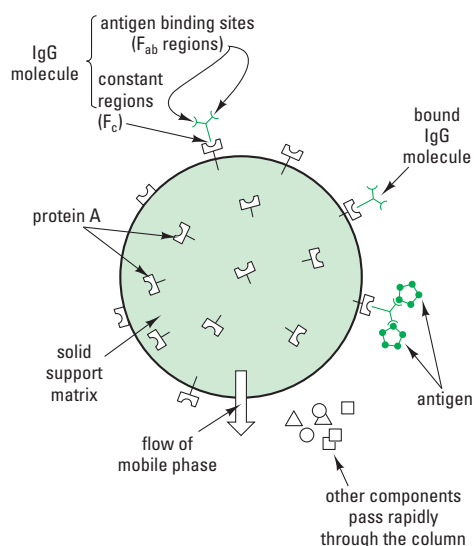


Fig. 48.12 Affinity chromatography using protein A as a ligand to bind IgG antibodies via the F_c region.

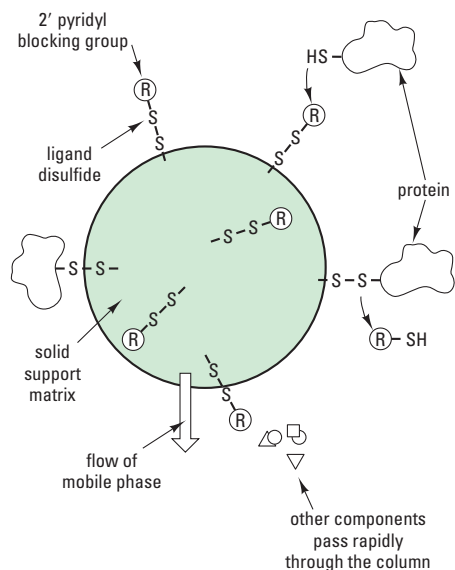


Fig. 48.13 Covalent chromatography. A protein with a free surface thiol group reduces the ligand disulfide, releasing the blocking group and forming a new disulfide.

Assaying analytes – remember that you cannot *quantify* a particular analyte without first *identifying* it: the presence of a single peak on a chromatogram does not prove conclusively that a single analyte is present.

- **Poly(U)-agarose**, an affinity support that you can use for the isolation of mRNA because of the biospecific hybridisation of poly(U) with the poly(A) ‘tail’ sequence characteristic of mRNAs. You can also use it to isolate proteins and enzymes that bind to RNA, such as reverse transcriptases.

Covalent chromatography

This is a variant of affinity chromatography which involves the formation of fairly strong, but reversible, covalent bonds between the affinity support and the molecule to be purified. One type of covalent chromatography is used for the purification of proteins containing thiol ($-SH$) groups (Fig. 48.13). If you apply the sample to a column containing matrix-attached disulfide 2'-pyridyl groups, thiol-containing proteins displace the 2'-pyridyl group on the support and become immobilised via disulfide bridges. You can then elute the bound components by including thiol reagents such as cysteine, glutathione, 2-mercaptoethanol or dithiothreitol (DTT) in the mobile phase. Another form of covalent chromatography uses immobilised boronic acid, which binds certain carbohydrate groups (for example, in glycoproteins).

Immobilised metal affinity chromatography (IMAC)

This exploits the ability of certain metal ions, especially Ni^{2+} , Cu^{2+} , Zn^{2+} , Hg^{2+} and Cd^{2+} , to bind to some proteins by forming coordination complexes with imidazole groups of histidine, indole groups of tryptophan, or thiol groups of cysteine residues. The metal ion may be immobilised by chelation with iminodiacetic acid covalently bound to agarose. Proteins that bind to the metal ions can be eluted using free metal-binding ligands (for example, amino acids) in the mobile phase.

A very useful application of IMAC is in the purification of ‘histidine-tagged’ recombinant proteins, for example, using pET plasmid vectors (see p. 732). The technique exploits the affinity of histidine-containing proteins for a chelating gel, typically Ni^{2+} immobilised on agarose. The histidine tag present at the *N*- or *C*-terminus of a recombinant protein forms complexes with the transition metal and is therefore bound to the gel. You can then desorb (elute) the proteins from the gel with increasing concentration of a substrate with an affinity for the chelated metal ion, for example, imidazole, using either a gradient or a step-wise approach. Alternatively, you can use chelating agents (for example, EDTA) or low pH for protein elution.

Optimising chromatographic separations

In any chromatographic technique, sample components leave the column at different elution volumes/different times, and are then monitored by a suitable detector (Chapter 49). The responses of the detector are recorded on a chart or a screen in the form of a *chromatogram*. Ideally the sample biomolecules will be completely separated and detection of components will result in a series of discrete individual peaks corresponding to each type of biomolecule (for example, Fig. 48.3). However, to minimise the possibility of overlapping peaks, or of peaks composed of more than one component, you will need to maximise the separation efficiency of the technique, which depends on:

- **selectivity**, as measured by the relative retention times of the two components, or by the volume of mobile phase between the peak

Separating small biomolecules – these will diffuse faster than large molecules, so they should be separated using faster flow rates.

Learning from experience – if you are unable to separate your biomolecule using a particular method, do not regard this as a failure, but instead, think about what this tells you about either the substance(s) or the sample.

maxima of the two components after they have passed through the column; this depends on the ability of the chromatographic method to separate two components with similar properties

- **band-broadening properties of the chromatographic system**, which influence the width of the peaks; these are mainly due to the effects of diffusion.

The separation efficiency, or resolution of two adjacent components, can be defined in terms of selectivity and peak broadening, using eqn [48.4]. In practical terms, good resolution is achieved when there is a large ‘distance’ (either time or volume) between peak maxima, and the peaks are as narrow as possible. The resolution of components is also affected by the relative amount of each substance: for systems showing low resolution, it can be difficult to resolve small amounts of a particular component in the presence of larger amounts of a second component. If you cannot obtain the desired results from a poorly resolved chromatogram, other chromatographic conditions, or even different methods, should be tried in an attempt to improve resolution. For liquid chromatography, you can make changes in the following factors to try to improve resolution:

- **Stationary phase particle size** – the smaller the particle, the greater the area available for partitioning between the mobile phase and the stationary phase. This partly accounts for the high resolution observed with HPLC and FPLC compared with low-pressure methods.
- **The slope of the salt gradient** in eluting IEC or HIC columns, for example, using computer-controlled adapted gradients.

In low-pressure liquid chromatography, the flow rate of the mobile phase must be optimised because this influences two band-broadening effects which are dependent on diffusion of sample molecules, i.e. (i) the flow rate must be slow enough to allow effective partitioning between the mobile phase and the stationary phase, and (ii) it must be fast enough to ensure that there is minimal diffusion along the column once the molecules have been separated. To allow for these opposing influences, you will need to find a compromise flow rate. If you prepare your own columns, they must be packed correctly, with no channels present that might result in uneven flow and eddy diffusion.

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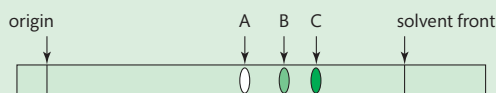
STUDY EXERCISES

48.1 Calculate R_F and R_X values from a chromatogram.

The figure represents the separation of three pigments by thin-layer chromatography.

- What is the R_F value of each pigment?
- What is the mobility of pigments B and C, relative to pigment A (R_A)?

Express both answers to three significant figures.



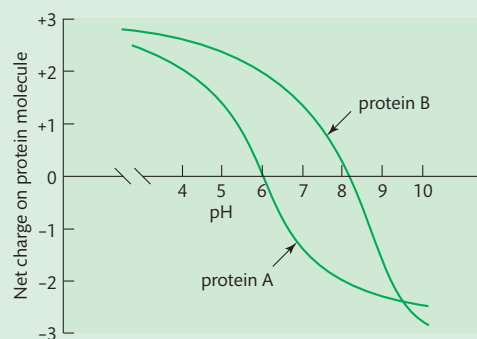
Thin-layer chromatographic separation of a mixture of three pigments (A, B and C).

48.2 Calculate the resolution and selectivity of two components from a chromatogram. Two compounds were separated by column chromatography, giving retention times of 4 min 30 s for A and 6 min 12 s for B, while a compound that was completely excluded from the stationary phase was eluted in 1 min 35 s. The base width of peak A was 40 s and the base width of peak B was 44 s. Calculate (a) the selectivity and (b) the resolution for these two compounds (express all answers to three significant figures).

48.3 Predict chromatographic behaviour on ion-exchange columns using protein titration curves.

The variation of net charge with pH for two proteins, A and B, is shown in the figure.

- What is the isoelectric point (pI) of each protein?
- If a mixture of these proteins was passed down an anion exchange column (for example, Q-Sepharose) equilibrated with buffer at pH 7.0, how would each protein behave in terms of elution and retention?
- If the proteins were bound to a cation exchanger (for example, S-Sepharose) at pH 5.5, predict the order in which the proteins would elute using a salt gradient.



48.4 Test your understanding of the action of ammonium sulfate on proteins. Explain why mobile phases containing a concentration of ammonium sulfate promote the binding of many proteins to HIC columns.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

49 Detecting and analysing compounds after chromatography

Definition

Flow cell – the sensor volume of a chromatographic detection system.

Once the individual components in a sample mixture have been separated by a given chromatographic technique (Chapter 48), a suitable detection system is required to monitor and record the elution of the components of the mixture as they pass through a ‘flow cell’.

KEY POINT The most appropriate detector depends on the type of chromatographic system and the application: ideally, the detector should be selected to show high sensitivity, a low detection limit and minimal noise or drift. These terms are defined in Chapter 56.

Understanding UV detection of proteins – note that the absorbance at 280 nm is mostly due to tryptophan and tyrosine residues; if a protein contains low amounts of these amino acids, its absorbance at this wavelength will be low.

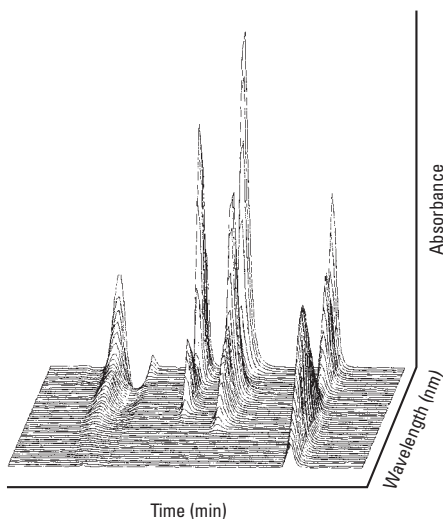


Fig. 49.1 Diode array detector absorption spectra of the eluent from an HPLC separation of a mixture of four steroids, taken every 15 s.

Overcoming interference with fluorescence detectors – you can use a dual flow cell to offset background fluorescence due to components of the mobile phase.

Using liquid chromatography detectors

UV/visible detectors

These have the advantages of versatility, sensitivity and stability, being of two types: fixed wavelength and variable wavelength. Fixed wavelength detectors are simple to use, with low operating costs. They usually contain a mercury lamp as a light source, emitting at several wavelengths between 254 and 578 nm; a particular wavelength is selected using suitable cut-off filters. The most frequently used wavelengths for analysis of biomolecules are 254 nm (for nucleic acids) and 280 nm (for proteins). Variable wavelength detectors use a deuterium lamp and a continuously adjustable monochromator for wavelengths of 190–600 nm. For both types of detector, sensitivity is in the absorbance range 0.001–1.0 (down to ≈ 1 ng), with noise levels as low as 4×10^{-5} . Note that sensitivity is partly influenced by the path length of the flow cell (typically 10 mm). Monitoring at short wavelength UV (for example, below 240 nm) may give increased sensitivity but decreased specificity, since many biological molecules absorb in this range. Additional problems with short wavelength UV detection include instrument instability, giving a variable baseline, and absorption by components of the eluting buffer (for example, TRIS, which absorbs at 206 nm).

An important development in chromatographic monitoring is diode array detection (DAD). The incident light comprises the whole spectrum of light from the source, which is passed through a diffraction grating and the diffracted light detected by an array of photodiodes. A typical DAD can measure the absorbance of each sample component at 1–10-nm intervals over the range 190–600 nm. This gives an absorbance spectrum for each eluting substance which may be used to identify the compound and give some indication as to its purity. An example of a three-dimensional diode array spectrum is shown in Fig. 49.1.

Fluorescence detectors

Many biomolecules, including some vitamins, nucleotides and porphyrins, show natural fluorescence (Table 46.1), or can be made to fluoresce by pre-column or post-column derivatisation with a fluorophore. Fluorescence detection is more sensitive than UV/visible detection (pp. 358–9), and may allow analysis in the picogram range. A fluorescence detector consists of a light source (for example, a xenon lamp), a diffraction grating to supply light at the excitation wavelength and a photomultiplier to monitor the

Maximising sensitivity with fluorescence detectors – the concentration of other sample components, e.g. pigments, must not be so high that they cause quenching of fluorescence.

Optimising electrochemical detection – the mobile phase must be free of any compounds that might give a response; all constituents must be of the highest purity.

emitted light (usually arranged to be at right angles to the excitation beam). Using an instrument with a laser light source can give an extremely narrow excitation waveband, resulting in increased sensitivity and specificity.

Electrochemical detectors

These offer very high sensitivity and specificity, with the possibility of detection of femtogram amounts of electroactive compounds such as catecholamines, vitamins, thiols, purines, ascorbate and uric acid. The two main types of detector, amperometric and coulometric, operate on similar principles, namely by measuring the change in current or potential as sample components pass between two electrodes within the flow cell. One of these electrodes acts as a reference (or counter) electrode (for example, a calomel electrode), while the other – the working electrode – is held at a voltage that is high enough to cause either oxidation or reduction of sample molecules. In the oxidative mode, the working electrode is usually glassy carbon, while in reductive mode a mercury electrode is used. In either case, a current flow between the electrodes is induced and detected.

Using gas chromatography detectors

Flame ionisation detectors (FID)

These are widely used detectors, being particularly useful for the analysis of a broad range of organic biomolecules. Detection involves passing the exit gas stream from the column through a hydrogen flame that has a potential of > 100 V applied across it (Fig. 49.2). Most organic compounds, on passage through this flame, produce ions and electrons that create a small current across the electrodes, and this is amplified for measurement purposes. The FID is very sensitive (down to ≈ 0.1 pg), with a linear response over a wide concentration range. One drawback is that the sample is destroyed during analysis.

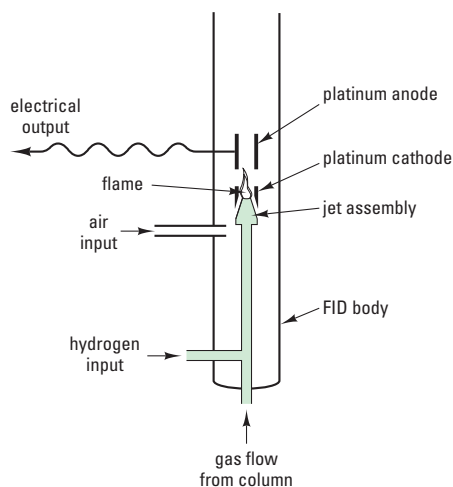


Fig. 49.2 Components of a flame ionisation detector (FID).

Thermal conductivity detectors (TCD)

These relatively simple detectors are based on changes in the thermal conductivity of the gas stream brought about by the presence of separated sample molecules. The detector elements are two electrically heated platinum wires, one in a chamber through which only the carrier gas flows (the reference detector cell), and the other in a chamber that takes the gas flow from the column (the sample detector cell). In the presence of a constant gas flow, the temperature of the wires (and therefore their electrical resistance) is dependent on the thermal conductivity of the gas. Analytes in the gas stream are detected by temperature-dependent changes in resistance that are dependent on the thermal conductivity of each separated molecule; the size of the signal is then directly related to concentration of the analyte.

The advantages of TCD include its applicability to a wide range of organic and inorganic biomolecules and its non-destructive nature, since the sample can be collected for further study. Its major limitation is its low sensitivity (down to ≈ 10 ng), compared with other systems.

Electron capture detectors (ECD)

These highly sensitive detectors (Fig. 49.3) are useful for the detection of certain compounds with electronegative functional groups, for example, halogens, peroxides and quinones. The gas stream from the column passes over a β -emitter (p. 406) such as ^{63}Ni , which provides electrons that cause

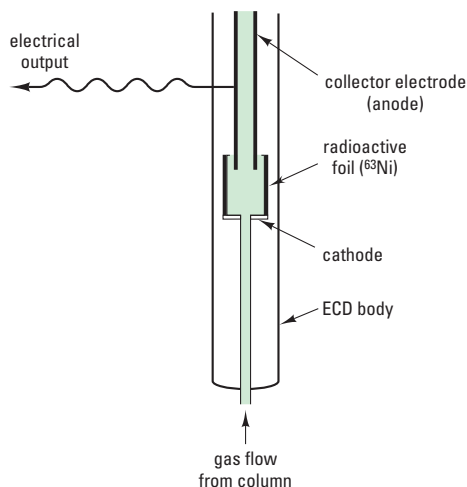


Fig. 49.3 Components of an electron capture detector (ECD).

Using chromatographic systems in conjunction with mass spectrometry – this approach has helped to identify and characterise hundreds of components present in complex biological systems, including flavour molecules in food, drug metabolites and pollutants in water.

ionisation of the carrier gas (for example, nitrogen). When carrier gas alone is passing the β -emitter, its ionisation results in a constant current flowing between two electrodes placed in the gas flow. However, when electron-capturing sample molecules are present in the gas flow, a decrease in current is detected. An example of the application of the ECD is in detecting and quantifying chlorinated pesticides.

Interfacing GC or HPLC with mass spectrometry (MS)

Mass spectrometry (pp. 412–14) used in conjunction with chromatographic methods can provide a powerful tool for identifying the components of complex mixtures, for example, aqueous pollutants. The procedure requires computer control of instruments and data storage/analysis (Fig. 49.4). One drawback is the limited capacity of the mass spectrometer – owing to its vacuum requirements – compared with the volume of material leaving the chromatography column. For capillary GC, the relatively small output can be fed directly into the ionisation chamber of a mass spectrometer. For packed column GC, a ‘jet separator’ is used to remove most of the carrier gas from the molecules to be analysed before they enter the mass spectrometer. Similarly, in HPLC, devices have been developed for solving the problem of large solvent volumes, for example, by splitting the eluent from the column so that only a small fraction reaches the mass spectrometer.

The computer-generated outputs from the mass spectrometer are similar to chromatograms obtained from other methods, and show peaks corresponding to the elution of particular components. However, it is then possible to select an individual peak and obtain a mass spectrum for the component in that peak to aid in its identification (p. 335).

Coupling capillary GC columns with FT-IR spectrometers (pp. 331–2) provides another powerful means of separating and identifying compounds in complex biological mixtures.

Recording and interpreting chromatograms

Recording detector output

For analytical purposes, the detector output is usually connected to a computer-based data analysis system with data acquisition hardware to convert the analogue detector signal to digital format, plus software to control the data acquisition process, store the signal information and display the resulting chromatogram. The software will also detect peaks

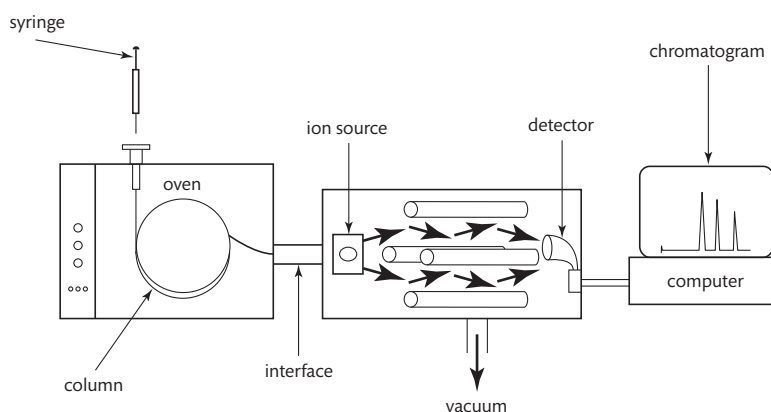


Fig. 49.4 Schematic diagram of a GC-MS instrument.

Interpreting chromatograms – *never* assume that a single peak is a guarantee of purity: there may be more than one compound with the same chromatographic characteristics.

Using a fraction collector – make sure you can relate individual fractions to the position of peaks on the chromatogram. Most fraction collectors send a signal to the recorder each time a fraction is changed.

and calculate their retention times and sizes (areas) for quantitative analysis. The software often incorporates functions to control the chromatographic equipment, enabling automatic operation. In sophisticated systems, the detector output may be compared with that from a ‘library’ of chromatograms for known compounds, to suggest possible identities of unknown sample peaks.

In simpler chromatographic systems, you may need to use a chart recorder for detector output. Two important settings must be considered before using a chart recorder:

- 1. The baseline reading** – this should be set only after a suitable quantity of mobile phase has passed through the column (prior to injection of the sample) and stability is established. Make sure that you set the chart recorder a little above the edge of the chart paper grid, to allow for any baseline drift.
- 2. The detector range** – you must select a range that ensures that the largest peaks do not go off the top of the chart. Adjust based on the expected quantity of analyte, or by trial-and-error. Use the maximum sensitivity that gives intact peaks. If peaks are still too large, you may need to reduce the amount of sample used, or prepare and reanalyse a diluted sample.

Interpreting chromatograms

Make sure you know the direction of the horizontal axis (usually, either volume or time) – it may run from right to left or vice versa – and make a note of the detector sensitivity on the vertical axis.

Ideally, the baseline should be ‘flat’ between peaks, but it may drift up or down owing to a number of factors, including:

- **air bubbles (in liquid chromatography)** (If the buffers used in the mobile phase are not effectively degassed, air bubbles may build up in the flow cell of the detector, leading to a gradual upward drift of the baseline, followed by a sharp fall when the accumulated air is released. Small air bubbles that do not become trapped may give spurious small peaks.)
- **changes in the composition of the mobile phase (for example, in gradient elution, p. 349)**
- **tailing of material from previous peaks**
- **carry-over of material from previous samples**; this can be avoided by efficient cleaning of columns between runs – allow sufficient time for the previous sample to pass through the column before you introduce the next sample
- **loss of stationary phase from the column** (column ‘bleed’), caused by extreme elution conditions.

Avoiding problems with air bubbles in liquid chromatography – always ensure that buffers are effectively degassed by vacuum treatment before use, and regularly clean the flow cell of the detector.

A peak close to the origin is likely to be caused by non-retained sample molecules, flowing at the same rate as the mobile phase, or to artefacts, for example, air (GC) or solvent (HPLC) in the sample. Whatever its origin, this peak can be used to measure the column dead time or column void volume (p. 348). No peaks from genuine sample components should appear before this peak.

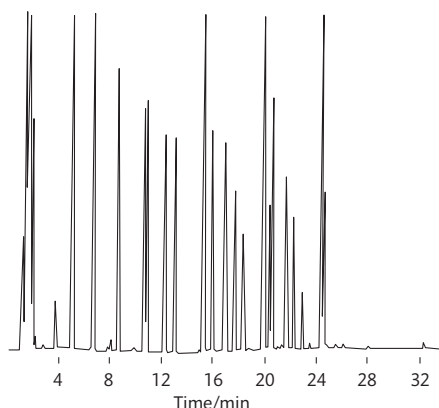


Fig. 49.5 A multicomponent chromatogram. Separation of many compounds, some that are well resolved, for example, peaks at 12–13 min and others that are not, for example, peaks at 24–25 min.

Using an external standard – you should analyse samples and standards more than once, to confirm the reproducibility of the technique.

Using an internal standard – you should add the internal standard to the sample at the first stage in the extraction procedure, so that any loss or degradation of test substance during purification is accompanied by an equivalent change in the internal standard, as long as the extraction characteristics of the internal standard and the test substance are very similar.

Quantifying biomolecules – note that quantitative chromatographic analysis often requires assumptions about the *identity* of separated components and that further techniques may be required to provide information about the nature of the biomolecules present, e.g. mass spectrometry (see Chapter 47).

Peaks can be denoted on the basis of their elution volume (used mainly in liquid chromatography) or their retention times (mainly in GC), as in Fig. 49.5. If the peaks are not narrow and symmetrical, they may contain more than one component. Where peaks are more curved on the trailing side compared with the leading side, this may indicate too great an association between the component and the stationary phase, or may result from overloading of the column.

Performing quantitative analysis

Most detectors and chemical assay systems give a linear response with increasing amounts of the test substance over a given ‘working range’ of concentration. Alternative ways of converting the measured response to an amount of substance are as follows:

- **External standardisation:** this is applicable where the sample volume is sufficiently precise to give reproducible results (for example, HPLC, column chromatography). You measure the peak areas (or heights) of known amounts of the substance to give a calibration factor or calibration curve which can be used to calculate the amount of test substance in the sample.
- **Internal standardisation:** here you add a known amount of a reference substance (not originally present in the sample) to the sample, to give an additional peak in the elution profile. You determine the response of the detector to the test and reference substances by analysing a standard containing known amounts of both substances, to provide a response factor (r), where

$$r = \frac{\text{peak area (or height) of test substance}}{\text{peak area (or height) of reference substance}} \quad [49.1]$$

Use this response factor to quantify the amount of test substance (Q_t) in a sample containing a known amount of the reference substance (Q_r), from the relationship:

$$Q_t = \frac{\text{peak area (or height) of test substance}}{\text{peak area (or height) of reference substance}} \times (Q_r/r) \quad [49.2]$$

Internal standardisation should be the method of choice wherever possible, since it is unaffected by small variations in sample volume (for example, for GC microsyringe injection). The internal standard should be chemically similar to the test substance(s) and must give a peak that is distinct from all other substances in the sample. An additional advantage of an internal standard that is chemically related to the test substance is that it may show up problems caused by changes in detector response, incomplete derivatisation, etc. A disadvantage is that it may be difficult to fit an internal standard peak into a complex chromatogram.

Sources for further study

Bouchonnet, S. (2013) *Introduction to GC-MS Coupling*. CRC Press, Boca Raton.

Cazes, J. (2021) *Encyclopedia of Chromatography*, 4th edn. CRC Press, Boca Raton.

Grob, R.L. and Barry, E.F. (2021) *Modern Practice of Gas Chromatography*, 5th edn. Wiley, New York.

McNair, H.M., Miller, J.M. and Snow, N.H. (2019) *Basic Gas Chromatography*, 3rd edn. Wiley, New York.

Sparkman, O.D., Penton, Z. and Kitson, F.G. (2011) *Gas Chromatography and Mass Spectrometry: A Practical Guide*, 2nd edn. Academic Press, Oxford.

Vitha, M. (2016) *Chromatography: Principles and Instrumentation*. Wiley, New York.

STUDY EXERCISES

49.1 Test your knowledge of detector terminology.

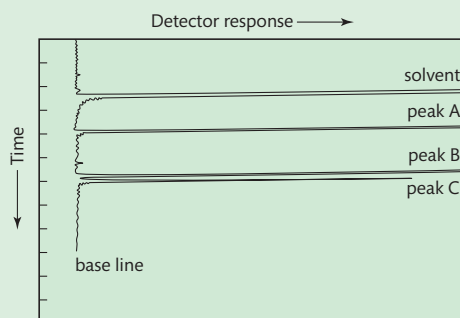
Explain what the following acronyms stand for:

- (a) FID;
- (b) TCD;
- (c) ECD;
- (d) DAD.

49.2 Check your knowledge of liquid chromatography detectors. Make a list of the various major types of liquid chromatography detectors in order, from highest to lowest sensitivity. Which of these methods is most versatile and why?

49.3 Consider how fluorescence detection can be applied to non-fluorescent molecules. Fluorescence detection of natural molecules is limited to molecules such as porphyrins, nucleotides and some vitamins, for example, riboflavin. What procedures can convert a non-fluorescent molecule into a form suitable for sensitive fluorescence detection? How does this increase the selectivity of the analysis?

49.4 Calculate the amount of substance in a chromatographic separation using an internal standard. The chromatogram shown in the figure on the right represents the separation of three carbohydrates by gas chromatography. Peak A corresponds to mannitol, peak B to sucrose and peak C to the internal standard, trehalose, at 1.50 mg. The results shown in the table were obtained for the retention times and areas of the three peaks.



Gas-liquid chromatographic separation of three carbohydrates (A, B and C). Note that the plotter shows peaks A and B to be off-scale; however, the instrument still gives a valid area measurement for these two peaks (see table).

Retention times and peak areas for carbohydrates A, B and C.

Peak	Retention time (min)	Area (relative)
A	3.92	2060
B	5.82	1898
C	6.03	604

Given a response factor (r) of 1.26 for mannitol and 0.92 for sucrose (relative to the internal standard), determine the amount of A and B in the sample (express your answer to three significant figures).

Answers to these study exercises are available at go.pearson.com/uk/he/resources

50 Methods of electrophoresis

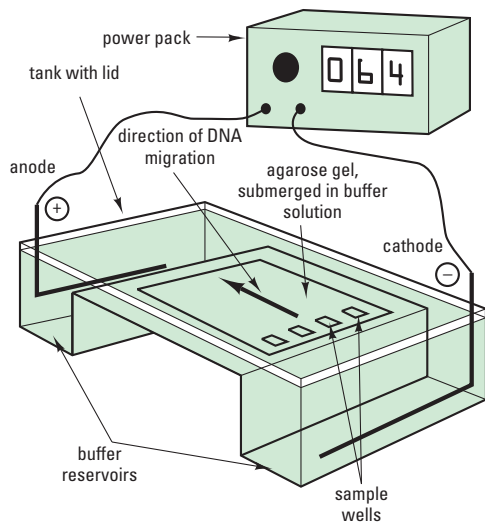


Fig. 50.1 Components of an electrophoresis system.

Electrophoresis is a separation technique based on the movement of charged molecules in an electric field. You are most likely to use electrophoresis for the isolation and purification of charged macromolecules such as nucleic acids and proteins, but techniques are also available for high resolution separations of smaller molecules such as amino acids (for example, by capillary electrophoresis, pp. 376–9).

The essential components of an electrophoretic system (Fig. 50.1) are:

- **two electrodes**, the anode and the cathode, between which a voltage can be applied using a microprocessor-controlled 'power pack' that provides control over voltage (V) and current (A) at constant power (W) for a specified time
- **a buffer (salt) solution**, through which the current flows and in which the ionic substances move at a controlled, constant pH
- **a supporting medium**, which limits diffusion and thermal convection, leading to enhanced and consistent separation of the charged molecules
- **a visualisation/detection system**, to view the results following electrophoresis.

This chapter deals mainly with the electrophoretic separation of proteins, which has many applications, including clinical diagnosis. However, the principles apply equally to other molecules (separation of nucleic acids is considered in Chapter 67).

KEY POINT The principle of electrophoretic separation is based on the fact that when an electric field is applied, Molecules of different size, shape and move at different rates and so the components of a mixture can be separated. This is, in essence, an incomplete form of electrolysis (p. 384), since the applied electrical field is switched off well before sample molecules reach the electrodes.

Understanding electrophoresis

The electrophoretic mobility of a charged molecule depends on the following:

- **Net charge** – negatively charged molecules (anions) migrate towards the anode (+), while positively charged molecules (cations) migrate towards the cathode (–); highly charged molecules move faster towards the electrode of opposite charge than those with lesser charge.
- **Size** – frictional resistance exerted on molecules moving in a solution means that smaller molecules migrate faster than large molecules.
- **Shape** – the effect of friction also means that the shape of the molecule will affect mobility, for example, globular proteins compared with fibrous proteins, linear DNA compared with circular DNA.
- **Electrical field strength** – mobility increases with increasing electrical potential (voltage), but there are practical limitations to using high voltages, especially because of heating effects.

Applying electrophoresis – this is a widely used technique, particularly for the analysis of complex mixtures and for the verification of purity (homogeneity) of isolated biomolecules.

Definitions

Electrical field strength – a measure of the magnitude of an electrical field, usually expressed in terms of electrical potential difference per unit length, as V m^{-1} .

Electrophoretic mobility – the rate of migration of a particular type of molecule in response to an applied electrical field.

Table 50.1 pK_a values of ionisable groups in selected amino acid residues of proteins

Group/residue	pK_a^*
Terminal carboxyl	3.1
Aspartic acid	4.4
Glutamic acid	4.4
Histidine	6.5
Terminal amino	8.0
Cysteine	8.5
Tyrosine	10.0
Lysine	10.0
Arginine	12.0

*Note that these are typical values: the pK_a will change with temperature and ionic strength. Acidic residues will tend to be negatively charged at pH values above their pK_a , while basic residues will tend to be positively charged below their pK_a .

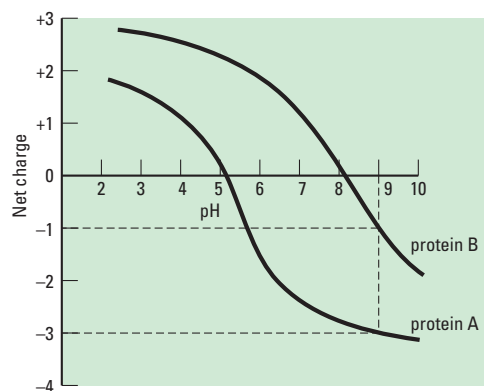


Fig. 50.2 Titration curves for two proteins, A and B, containing different proportions of acidic and basic amino acid residues.

The combined influence of net charge and size means that mobility (μ) is determined by the charge: density ratio or the charge: mass ratio, according to the formula:

$$\mu = \frac{qE}{r} \quad [50.1]$$

where q is the net charge on the molecule, r is the molecular radius and E is the field strength.

Separating proteins using electrophoresis

As noted above, the net charge of a sample molecule determines its direction of movement and significantly affects its mobility. The net charge of a protein molecule is pH-dependent, and is determined by the relative numbers of positively and negatively charged amino acid side chains at a given pH (Table 50.1). The degree of ionisation of each amino acid side chain is pH-dependent, resulting in a variation of net charge on the protein at different pH values (Fig. 50.2). Since an individual protein will have a unique content of ionisable amino acids, each protein will have a characteristic 'titration curve' when net charge is plotted against pH. Thus, electrophoresis is always carried out at *constant* pH and a suitable buffer must be present along with the sample to maintain that pH (Chapter 24). If the proteins shown in Fig. 50.2 were subjected to electrophoresis at pH 9.0, and if the proteins were of similar size and shape, then the rate at which protein A (net charge, -3) migrates towards the anode would be faster than that for protein B (net charge, -1). Separation of proteins is usually carried out at alkaline pH, where most proteins carry a net negative charge.

Using standard methods in electrophoresis

Most types of electrophoresis using supporting media (described below) are simple to carry out and the apparatus can be easily constructed, although inexpensive equipment is commercially available. High resolution techniques such as 2D electrophoresis and capillary electrophoresis require more sophisticated equipment, both for separation and analysis (Chapter 51).

Basic apparatus

You can perform simple electrophoretic separations either horizontally (Fig. 50.1) or vertically (Fig. 50.3). The electrodes are normally made of platinum wire, each in its own buffer compartment. In vertical electrophoresis, the buffer solution forms the electrical contact between the electrodes and the supporting medium in which the sample separation takes place. In horizontal electrophoresis, electrical contact can be made by buffer-soaked paper 'wicks' dipping in the buffer reservoir and laid upon the supporting medium. The buffer reservoir normally contains a divider acting as a barrier to diffusion (but not to electrical current), so that localised pH changes which occur in the region of the electrodes (as a result of electrolysis, p. 384) are not transmitted to the supporting medium or the sample. You 'spot' individual samples onto a solid supporting medium containing buffer or apply them to 'wells' formed in the supporting medium. The power pack used for most types of electrophoresis should be capable of delivering $\approx 500 \text{ V}$ and $\approx 100 \text{ mA}$.

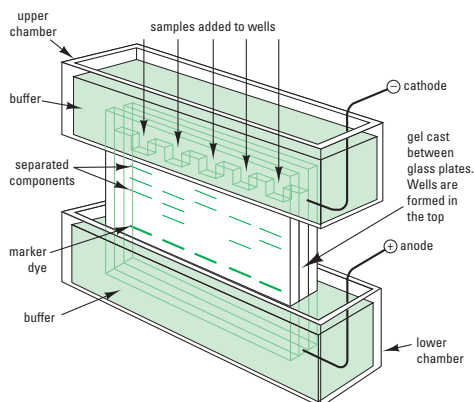


Fig. 50.3 Apparatus for vertical slab electrophoresis (components move downwards from wells, through the gel matrix).

Minimising diffusion – make your sample zone as narrow as possible, and fix and stain protein bands as soon as possible after the run.

Definition

Ohm's law $V = IR$, where V = voltage, I = current and R = resistance.

Optimising electrophoresis – attempting to minimise heat production using very low currents is not practical, since it leads to long separation times, and therefore to increased diffusion.

Definition

Electro-osmotic flow – the osmotically driven mass flow of water resulting from the movement of ions in an electrophoretic system.

Choosing an appropriate supporting medium

You can minimise the effects of convection currents (resulting from the heating effect of the applied field) and the diffusion of molecules within the buffer solution by carrying out the electrophoresis in a porous supporting medium. This contains buffer electrolytes and the sample is added in a discrete location or zone. When you apply the electrical field, individual sample molecules remain in sharp zones as they migrate at different rates. After separation, you can avoid post-electrophoretic diffusion of selected biomolecules (for example, proteins) by 'fixing' them in position on the supporting medium, for example, using trichloroacetic acid (TCA).

The heat generated during electrophoresis is proportional to the square of the applied current and to the electrical resistance of the medium: even when a supporting medium is used, you may find that heat production will lead to zone broadening by increasing the rate of diffusion of sample components and buffer ions. Heat denaturation of sample proteins may also occur, resulting in loss of biological activity, for example, with enzymes (p. 464). Another problem is that heat will reduce buffer viscosity, leading to a decrease in resistance. If the electrophoresis is run at constant voltage, Ohm's law dictates that, as resistance falls, the current will increase, leading to further heat production. You can avoid this by using a power pack that provides constant power. In practice, most electrophoresis equipment incorporates a cooling device; even so, distortions of an electrophoretic zone from the ideal 'sharp, linear band' can often be explained by inefficient heat dissipation.

Your choice of supporting medium can be subdivided into:

- **inert media** – these provide physical support and minimise convection: separation is based on charge density only (for example, cellulose acetate)
- **porous media** – these introduce molecular sieving as an additional effect: their pore size is of the same order as the size of molecules being separated, restricting the movement of larger molecules relative to smaller ones. Thus, separation depends on both the charge density and the size of the molecule.

With some supporting media, for example, cellulose acetate, you may observe a phenomenon called electro-endosmosis or electro-osmotic flow (EOF). This occurs because of the presence of negatively charged groups on the surface of the supporting medium, attracting cations in the electrophoresis buffer solution and creating an electrical double layer. The cations are hydrated (surrounded by water molecules) and when the electric field is applied, they are attracted towards the cathode, creating a flow of solvent that opposes the direction of migration of anionic biomolecules towards the anode. The EOF can be so great that weakly anionic biomolecules (for example, antibodies) may be carried towards the cathode.

Where necessary, you can avoid EOF by using supporting media such as agarose or polyacrylamide (p. 367), but it is not always a hindrance to electrophoretic separation. Indeed, the phenomenon of EOF is used in the high-resolution technique of capillary electrophoresis (pp. 376–7).

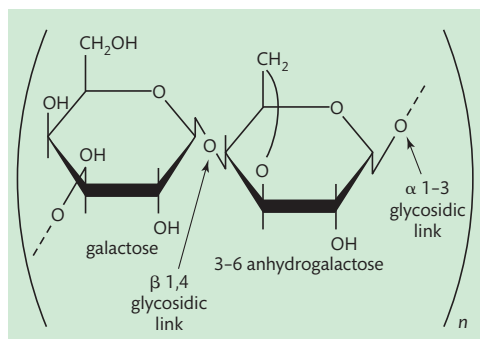


Fig. 50.4 Structure of agarose. Additional sulfate and pyruvyl groups are attached at selected hydroxyls in the polymer.

Handling cellulose acetate – you must handle the fragile strips with care, avoiding touching the flat surfaces with your fingers.

Taking advantage of the properties of polyacrylamide gels – in addition to their versatility in terms of pore size, these gels are chemically inert, stable over a wide range of pH, ionic strength and temperature, and transparent.

SAFETY NOTE When preparing gels for PAGE, note that both acrylamide and bisacrylamide are extremely potent neurotoxins, so you must wear plastic gloves when handling solutions containing these reagents. Although the polymerised gel is nontoxic, it is still advisable to wear gloves when handling the gel, because some monomer may still be present.

Cellulose acetate Acetylation of the hydroxyl groups of cellulose produces a less hydrophilic structure than cellulose in the form of paper: as a result it holds less water and diffusion is reduced, with a corresponding increase in resolution. Cellulose acetate is often used in the electrophoretic separation of plasma proteins in clinical diagnosis – it can be carried out quickly (≈ 45 min) and its resolution is adequate to detect gross differences in various types of protein (for example, paraproteins in myeloma). Cellulose acetate has a fairly uniform pore structure and the pores are large enough to allow unrestricted passage of all but the largest of molecules as they migrate through the medium.

Agarose Agarose is the neutral, linear polysaccharide component of agar (from seaweed), consisting of repeating galactose and 3,6-anhydrogalactose subunits (Fig. 50.4). To create an agarose gel, you mix powdered agarose with electrophoresis buffer at concentrations of 0.5–3.0% w/v, boiled until the mixture becomes clear, poured onto a glass plate, then allowed to cool until it solidifies. Gelation is due to the formation of hydrogen bonds both between and within the agarose polymers, resulting in the formation of pores. The pore size depends on the agarose concentration. Low concentrations produce gels with large pores relative to the size of proteins, allowing them to migrate relatively unhindered through the gel, as determined by their individual charge densities. Low concentrations of agarose gel are suitable for techniques such as immunoelectrophoresis (p. 395) and isoelectric focusing (p. 375), where charge is the main basis of separation. The smaller pores produced by higher concentrations of agarose may result in molecular sieving.

When agarose gels are used for the separation of DNA, the large fragment size means that molecular sieving is observed, even with low concentration gels. This is the basis of the electrophoretic separation of nucleic acids (Chapter 67).

Polyacrylamide Polyacrylamide gel electrophoresis (PAGE) plays a major role in protein analysis, both for one-dimensional and two-dimensional separations. The gel is formed by polymerising acrylamide monomer into long chains and cross-linking these chains using *N,N*'-methylene bisacrylamide (often abbreviated to 'bis'). The chemical process is shown in Fig. 50.5. In most protocols, polymerisation is initiated by free radicals produced by ammonium persulfate in the presence of *N,N,N*',*N*'-tetramethylethylenediamine (TEMED). The photodecomposition of riboflavin can also be used as a source of free radicals.

The formation of polyacrylamide from its acrylamide monomers is extremely reproducible under standard conditions, and electrophoretic separations are correspondingly precise. The pore size, and hence the extent of molecular sieving, depends on the total concentration of monomer (%T), i.e. acrylamide plus bisacrylamide in a fixed ratio. This means that pores in the gel can be 'tailored' to suit the size of biomolecule to be separated: gels containing 3% acrylamide have large pores and are used in methods where molecular sieving should be avoided (for example, in isoelectric focusing, p. 375), while higher concentrations of acrylamide (5–30%T) introduce molecular sieving to various degrees depending on the size of the sample components, i.e. with 30% acrylamide gels, molecules as small as M_r 2000 may be subject to molecular sieving. Gels of 6.25% are necessary for molecular sieving of molecules of M_r 7×10^6 , but such gels are almost fluid

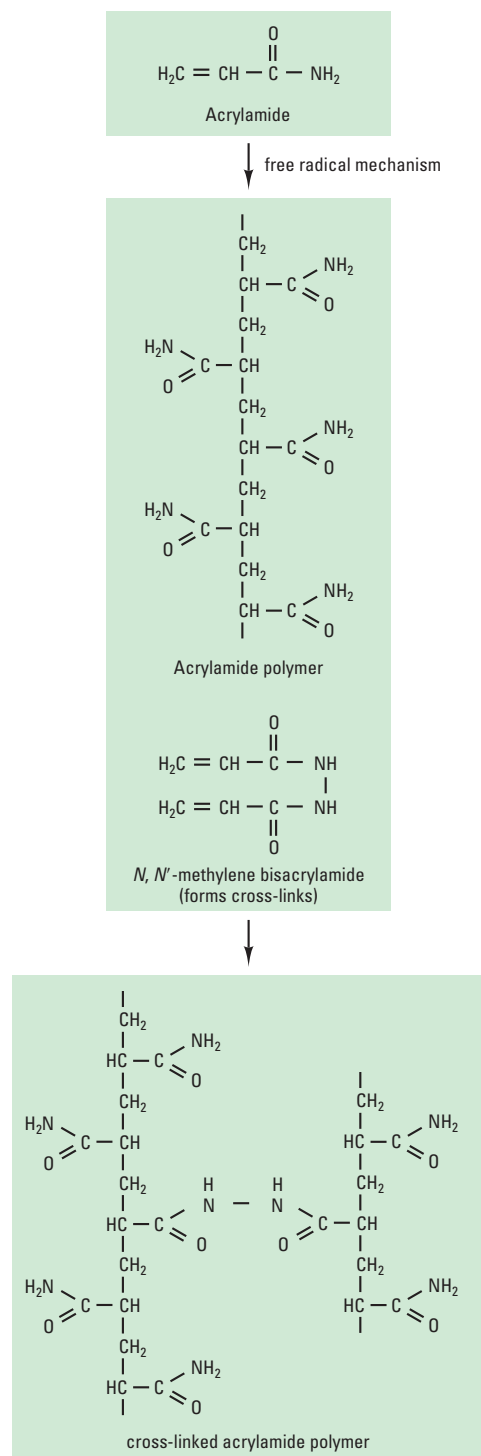


Fig. 50.5 Reactions involved in the formation of polyacrylamide gels.

and require 0.5% agarose to make them solid. Note that a gel of 3% will separate DNA by molecular sieving, owing to the large size of the nucleic acid molecules (p. 514).

Before you embark on a particular PAGE protein separation, you will need to think about the general strategy for that separation and make certain choices, including whether to use the following:

- Rod or slab gels** – flat slab gels are formed between glass plates, using plastic spacers 0.75–1.5 mm thick: rod gels are made in narrow bore tubes. For most separations using several samples, a slab gel saves time because up to 25 samples can be separated under identical conditions in a single gel, while rod gels can only be used for individual samples. Rectangular slab gels are also easier to read, by densitometry, and photography. However, you may find rod gels useful in preliminary separations, for determining a suitable pH and gel concentration, and for applications where the gel is sliced to extract and assay proteins of interest.
- Dissociating or non-dissociating conditions** – the most widely used PAGE protein separation technique uses an ionic detergent, usually sodium dodecyl sulfate (SDS), which dissociates proteins into their individual polypeptide subunits and gives a uniform net charge along each denatured polypeptide. This technique, known as SDS-PAGE, requires only microgram amounts of sample and is quick and easy to carry out. On the other hand, if it is necessary to preserve the native protein conformation and biological activity, non-dissociating conditions are used, i.e. no SDS is added. In SDS-PAGE the sample protein is normally heated to 100 °C for 2 min, in buffer containing 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol, the latter to cleave any disulfide bonds. The resultant polypeptides bind to SDS in a constant weight ratio, with 1.4 g of SDS per gram of protein. As a result, the intrinsic net charge of each polypeptide is ‘swamped’ by the negative charge imposed by SDS, and there is a uniform negative charge per unit length of polypeptide. Since the polypeptides now have identical charge densities, when they are subject to PAGE (with SDS present) using a gel of appropriate pore size, molecular sieving will occur and they will migrate strictly according to polypeptide size. This not only gives effective separation, but you can determine the molecular mass of a given polypeptide by comparing its mobility to polypeptides of known molecular mass run under the same conditions (Fig. 50.6). Several manufacturers (for example, Pharmacia, Sigma) supply molecular mass standard kits which may include polypeptides of M_r 11 700 to 212 000 (Table 50.2), together with details of their preparation and use. Where necessary, you can concentrate the treated sample by ultrafiltration and alter the buffer composition by diafiltration (p. 456).
- Continuous or discontinuous buffer systems** – a continuous system is where the same buffer ions are present in the sample, gel and buffer reservoirs, all at the same pH. You load the sample directly onto a gel (the ‘separating gel’ or ‘resolving gel’) that has pores small enough to introduce molecular sieving. In contrast, discontinuous systems have different buffers in the gel compared to the reservoirs, both in terms of buffer ions and pH. Here, you load the sample onto a large-pore ‘stacking gel’, previously polymerised on top of a small-pore separating

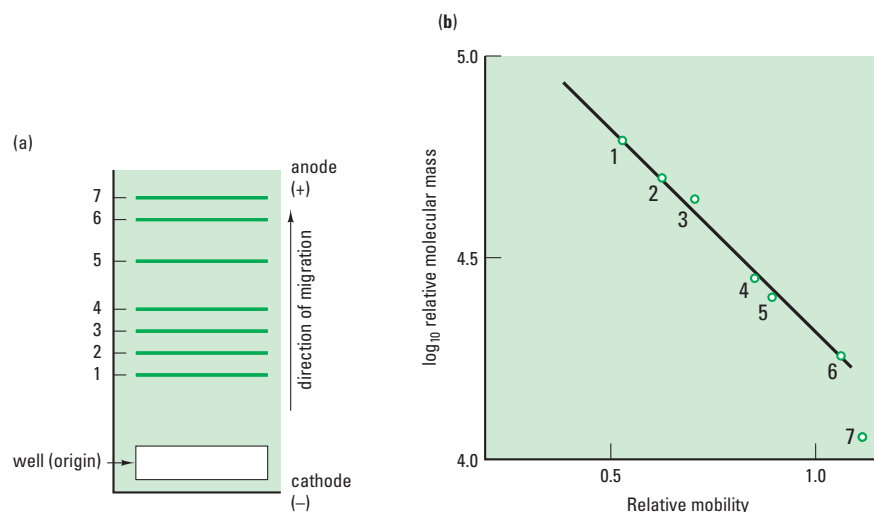


Fig. 50.6 Determination of relative molecular mass (M_r) of proteins by SDS-PAGE. (a) Mixture of: 1 cytochrome c, 2 myoglobin, 3 g-globulin, 4 carbonic anhydrase, 5 ovalbumin, 6 albumin, 7 transferrin. (b) Plot of $\log M_r$ against distance travelled through gel.

Preparing polyacrylamide gels – most solutions used for gel preparation can be made in advance, but the ammonium persulfate solution must be prepared immediately before use.

Selecting and using gels in 2D electrophoresis – a rod gel may be used for the first dimension and a slab gel for the second dimension.

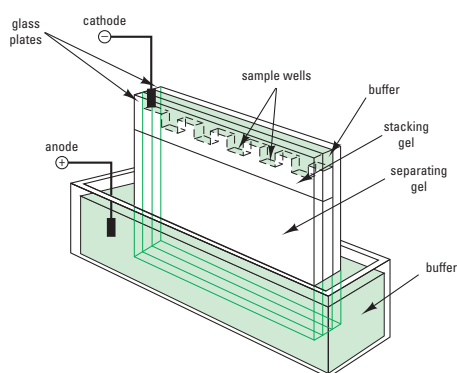


Fig. 50.7 Apparatus for discontinuous electrophoresis.

gel (Fig. 50.7). The individual proteins in the sample concentrate into very narrow zones during their migration through the large-pore gel and stack up according to their charge densities, prior to separation in the small-pore gel, giving enhanced results compared with continuous systems.

- **pH and buffer for the separation** – You can carry out PAGE between pH 3 and 10. The pH is not critical for continuous SDS-PAGE, since SDS-treated polypeptides are negatively charged over a wide pH range. However, when using non-dissociating systems, the pH can be critical, particularly if the biological activity of the molecules is to be retained.
- **Gel concentration** – at one extreme, a gel with a very high percentage T might totally exclude the sample components, while a gel with a very low percentage T might lead to all SDS-treated proteins migrating at the same rate, i.e. with the electrophoretic front. A sensible approach is to set up a series of rod gels in the range 5–15% T and observe the separation and resolution obtained. Alternatively, you can use a *gradient gel*, in which the percentage T increases, and hence pore size decreases, in the direction of protein migration. A useful gradient for a preliminary experiment would be 5–20% T. Such gels are able to resolve protein mixtures with a wide range of molecular masses. Furthermore, as proteins migrate into regions of ever-decreasing pore size, the movement of the leading edge of a zone will become increasingly restricted. This allows the trailing edge to catch up, resulting in considerable zone sharpening. Gradient formers are fairly simple to make, and are commercially available.

Practical details of the preparation of PAGE and SDS-PAGE gels are given in Table 50.3, while Box 50.1 gives the procedure for SDS-PAGE separation of proteins (see Westermeier, 2016, or Kurien and Schofield, 2012 for further details).

Table 50.2 Molecular masses of standard proteins used in electrophoresis

Protein	M_r	$\log_{10} M_r$
Cytochrome c	11 700	4.068
Myoglobin	17 200	4.236
γ -globulin (light chain)	23 500	4.371
Carbonic anhydrase	29 000	4.462
Ovalbumin	43 000	4.634
γ -globulin (heavy chain)	50 000	4.699
Human albumin	68 000	4.832
Transferrin	77 000	4.886
Myosin (heavy chain)	212 000	5.326

Following the progress of PAGE – add bromophenol blue solution (0.002% w/v) to the sample in the ratio 1:25 (dye: sample). This highly ionic, small M_r dye migrates with the electrophoretic front.

Choosing a buffer system for PAGE – discontinuous systems are more time-consuming to prepare, but have the advantage over continuous systems in that relatively large volumes of dilute sample can be used and good resolution is still obtained.

Carrying out post-electrophoretic procedures

For protein electrophoresis, the following stages are appropriate: details for nucleic acids are given on pp. 513–15.

Handling the supporting medium

You must handle all types of supporting medium carefully: wearing gloves is advisable, for safety and to avoid transfer of proteins from the skin. You should transfer cellulose acetate strips immediately to a fixing and staining solution. You should dry agarose gels quickly before staining. To free polyacrylamide gels in vertical slabs, carefully separate them from one of the glass plates in which they are formed, taking care to lever the glass at a point well away from the part of the gel containing the wells: once free, you should immediately transfer the gels to fixing or staining solution.

You can recover rod gels by a process called ‘rimming’, a technique that takes a little practice to master. To remove the gel from the tube, hold the tube in one hand and, using a syringe with a long blunt needle, squirt water between the gel and the inner wall of the tube: while squirting, move the needle gently up and down and rotate the tube. If the gel does not become free, perform the same procedure at the other end of the tube, until the gel is released. If necessary, a Pasteur pipette bulb applied to one end can be used to push the gel out of the tube.

Fixing, staining and destaining

To prevent separated proteins from diffusing, you would normally fix them in position. Proteins on cellulose acetate strips can be fixed and stained using Ponceau S (0.2% w/v in a solution of 3% v/v aqueous trichloroacetic acid, TCA).

Fixing is also required for most types of gel electrophoresis: 3% v/v TCA is often used. The most widely used stain for protein separations in gels is Coomassie Blue R-250 (where R = reddish hue): the detection limit is $\approx 0.2 \mu\text{g}$ and staining is quantitative up to $20 \mu\text{g}$ for some proteins. It is normal for background staining of the gel to occur, and removal of background colour (‘destaining’) can be achieved either by diffusion or

Table 50.3 Preparation of gels for PAGE and SDS-PAGE. The gel solutions are made by mixing the components in the proportions and in the order shown. Figures are mL of each solution required to give the stated % gel strength

Solution (added in order shown)	PAGE			SDS-PAGE		
	3.5% gel (T = 3.6%)	5% gel (T = 5.1%)	7.5% gel (T = 7.7%)	5% gel (T = 5.1%)	7.5% gel (T = 7.7%)	10% gel (T = 10.2%)
1. Distilled water	19.3	14.9	7.5	14.9	7.5	–
2. TRIS-glycine buffer, pH 8.9, 0.1 mol L ⁻¹	33.0	33.0	33.0	–	–	–
3. Imidazole buffer, pH 7.0, 0.1 mol L ⁻¹ plus 0.2% w/v SDS	–	–	–	33.0	33.0	33.0
4. Acrylamide solution 22.2% w/v and 0.6% w/v bis	10.4	14.8	22.2	14.8	22.2	29.7
5. Ammonium persulfate solution, 0.15% w/v	3.2	3.2	3.2	3.2	3.2	3.2
6. TEMED	<u>0.1</u>	<u>0.1</u>	<u>0.1</u>	<u>0.1</u>	<u>0.1</u>	<u>0.1</u>
Final volume (mL)	66.0	66.0	66.0	66.0	66.0	66.0

Box 50.1 How to carry out SDS-PAGE for protein separation

- 1. Prepare the gel.** Nowadays, many laboratories use pre-cast gels, bought from a manufacturer (e.g. Bio-Rad). However, if you are preparing your own gel, you will need to follow the protocol very carefully. Typically the correct proportions of acrylamide, bisacrylamide and SDS are mixed together and degassed, under vacuum. Then ammonium persulphate and tetramethylethylenediamine (TEMED) are added to trigger the polymerisation. Once the latter two constituents are added, the gel should be poured immediately into the casting tray, with the well former 'comb' in position.
- 2. Prepare the samples.** Each protein sample is mixed with a buffer solution containing SDS (to bind to the dissociated proteins), plus dithiothreitol (DTT) (to cleave disulphide bonds in the proteins) and a 'tracker' dye (e.g. bromophenol blue), then heated for 5 min at 95 °C, to disrupt the tertiary structure and convert the polypeptide chains to linear form.
- 3. Load the samples on to the gel.** Add individual samples to the wells using a pipettor (p. 143). The volume of sample added to each well is typically less than 100 µL, so a very steady hand and careful dispensing are needed to accurately pipette each sample (steady the pipettor using your second hand). To optimise the separation of proteins, keep the sample volume as small as possible.
- 4. Load the molecular mass standards.** Nowadays, many labs use 'rainbow' markers, with a wide range of proteins of known molecular mass, each of which is stained a different colour, to enable estimation of the molecular masses of unknown proteins, by visual comparison and interpolation (Fig. 50.6).
- 5. Run the electrophoresis.** Position the gel with the well/samples closest to the cathode (negative electrode), since they will move towards the anode during electrophoresis as a result of their negative charge. Protein separation is typically carried out at 80–100 V for 1–2 h (see manufacturer's instructions for specific details, according to which 'power pack' you are using): the gel should be run until the tracking dye has migrated across 80% of the gel). Higher voltages give faster separation, but poorer separation of proteins and may cause denaturation due to heating.
- 6. Fix and stain the gel.** One of the most widely used approaches uses Coomassie Brilliant Blue stain. Typically, this involves immersing the gel in 0.25% w/v Coomassie Blue for 1 h then destaining overnight in a methanol/acetic acid solution. A safer, water-based alternative is available (e.g. Bio-Rad Bio-Safe Coomassie Stain), allowing the gel to be destained in water overnight. After destaining, separated proteins are visible as blue bands against an unstained background. For higher resolution and greater sensitivity, a silver stain can be used instead of Coomassie Brilliant Blue.
- 7. Examine the results.** After destaining, separated proteins are visible as blue bands against an unstained background (Fig. 50.6(a)). The position of these bands can be compared with the molecular mass standards to determine the size of each band in the test sample. For greater accuracy, the distance moved by the molecular mass standards can be plotted against \log_{10} relative molecular mass and the resulting relationship (Fig. 50.6(b)) used to determine the size of unknown bands.

Choosing a pH for protein

electrophoresis – many proteins have isoelectric points in the range pH 4–7 and in response to electrophoresis with buffers in the region pH 8.0–9.5, most proteins will migrate towards the anode.

Separating protein mixtures – for high resolution, a combination of dissociating and discontinuous PAGE in slab gels is the system of choice.

electrophoresis. To destain by diffusion, transfer the gel to isopropanol:acetic acid:water (12.5 : 10: 77.5 v/v/v) and allow to stand for 48 h, or change the solution several times to speed up the staining process. Electrophoretic destaining can remove Coomassie Blue, which is anionic: stained gels are placed between porous plastic sheets with electrodes on each side, and the tank is filled with 7% acetic acid. Passing a current of up to 1.0 A through the gel for around 30–40 min. should result in substantial destaining.

If you need greater sensitivity (for example, for nanogram to femtogram amounts), or when using high resolution techniques such as 2D electrophoresis (p. 376), silver staining can be used. Depending on the protocol chosen and the proteins being stained, the silver technique can be fivefold to 200-fold more sensitive than Coomassie Blue. The method involves a fixation step (for example, with TCA), followed by exposure to silver nitrate solution and development of the stain. The silver

Deciding what to do if your polyacrylamide gels fail to set –

polymerisation is inhibited by oxygen, so solutions should be degassed, and the surfaces of the polymerisation mixture exposed to air should be overlaid with water; if your gels still do not polymerise, the most common cause is the use of 'old' ammonium persulfate stock solution. If low pH buffers are used, polymerisation may be delayed because TEMED is required in the free base form.

Handling gels – avoid touching gels with paper as it sticks readily and is difficult to remove without tearing.

Avoiding overloaded gels and band distortion – determine the protein concentration of the sample beforehand. Around 100 µg of a complex mixture, or 1–10 µg of an individual component, will be sufficient, but bear in mind that underloading may result in bands being too faint to be detected.

Optimising resolution – keep the sample volume as small as possible; methods for concentrating protein solutions are considered on pp. 454–6. For vertical slab gels and for rod gels, include 10% w/v sucrose or glycerol to increase density and allow buffer solution to be overlaid on the sample without dilution.

Understanding the terminology of high purity water – this is usually produced by reverse osmosis and its purity is often expressed with respect to its resistivity: ultrapure water has a resistivity of $18 \text{ M}\Omega \text{ cm}^{-1}$ (informally referred to as 'eighteen megohm water').

ions are thought to react with basic and thiol groups in proteins, and subsequent reduction (for example, by formaldehyde at alkaline pH, or by photodevelopment) leads to deposits of silver in the protein bands. Most proteins stain brown or black, but lipoproteins may stain blue, and some glycoproteins stain yellow or red. Some proteins lacking in amino acids with reducing groups (for example, those lacking cysteine residues) may stain negatively, i.e. the bands are more transparent than the background staining of the gel. Although many protocols have been published, silver stain kits are commercially available, for example, from Bio-Rad.

Although silver staining has clear advantages in terms of sensitivity, for routine work it is more laborious and expensive to carry out than the Coomassie Blue method. It also requires high purity water, otherwise significant background staining occurs. Another feature is that the staining can be non-specific, since DNA and polysaccharides may stain on the same gel as proteins.

Other methods for the detection of separated components include:

- **autoradiography** for proteins labelled with ^{32}P or ^{125}I (p. 407)
- **fluorescence** for proteins pre-labelled with fluorescent dyes (p. 426)
- **periodic acid-Schiff (PAS) stain** using dansyl hydrazine for glycoproteins.

Blotting

Here, the term 'blotting' refers to the transfer of separated proteins from the gel matrix to a thin sheet such as nitrocellulose membrane (commercially available from, for example, Millipore, Amersham). The proteins bind to this membrane, and are immobilised. Blotting of proteins is usually achieved by electrophoretic transfer, and this process is normally referred to as Western blotting (see also Southern blotting and Northern blotting for DNA and RNA, respectively, pp. 516–17). Its major advantage is that the immobilised proteins on the surface of the membrane are readily accessible to detection reagents, and staining and destaining can be achieved in less than 5 min. Use of labelled antibodies to detect specific proteins (immunoblotting) can take less than 6h. In addition, it is easy to dry and store Western blots for long periods, for further analysis.

Detection of enzymes

If you need to detect enzyme activity you should use a non-denaturing gel. The gel matrix will hinder diffusion of the enzyme, but will allow access to the small molecular weight substrates, co-factors and dyes necessary to localise enzyme activity *in situ*. Most methods for detecting enzymes on gels are modifications of protocols originally developed by histochemists, for example:

- **NAD^+ -requiring oxidoreductases** can be detected by incubating the gels with substrate, NAD^+ and a solution of a tetrazolium salt which, when reduced, forms an insoluble coloured formazan dye
- **transferases** and **isomerases** can be detected by coupling their reactions to an oxidoreductase-requiring reaction, visualised as described above

Naming of blotting technique – following the description of ‘Southern’ blotting of DNA by Dr Ed Southern (p. 516), other points of the compass have been used to describe other forms of blotting, with ‘Western’ blotting for proteins.

Example For detection of lactate dehydrogenase (LDH): when a solution containing lactate, NAD^+ , phenazine methasulfate (PMS) and methyl thiazolyl tetrazolium (MTT) is added to a gel containing LDH, a series of redox reactions occurs in the enzyme-containing regions, starting with the oxidation of lactate to pyruvate, and proceeding via NAD^+ , PMS and MTT to the eventual reduction of MTT to formazan dye. After incubation at 37 °C in the dark (since MTT is light-sensitive), LDH is detected by the appearance of blue-black bands on the gel.

- **hydrolases** can be detected using appropriate chromogenic or fluorogenic substrates
- **phosphatases** can be detected by precipitating any phosphate released from the substrate with Ca^{2+} .

For more details, see Manchenko (2019).

Recording and quantification of results

A number of expensive, dedicated instruments are available for the analysis of gels, for example, laser densitometers. Alternatively, gel scanning attachments can be purchased for spectrophotometers, allowing measurement and recording of the absorbance of the Coomassie Blue stained bands at 560–575 nm: for instruments connected to a computer, quantification of individual components can be achieved by integrating the areas under the peaks.

You can photograph gels using a digital camera, a conventional camera (use fine grain film), or using a photocopier. Alternatively, a dedicated image capture and analysis system may be used, for example, GelDoc. The gel should be placed on a white glass transilluminator. A red filter will increase contrast with bands stained with Coomassie Blue. If the gels themselves need to be retained, they can be preserved in 7% acetic acid. Alternatively, they can be dried using a commercially available gel dryer.

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STUDY EXERCISES

50.1 Find out why the net charge on a protein molecule varies with pH. Identify the amino acids primarily responsible for determining the net charge on a protein molecule and draw simple diagrams to represent the ionisation of their side chains, indicating how you would expect these side chains to be charged at acid, neutral and alkaline pH values.

50.2 Explain the function of the various reagents used in SDS-PAGE analysis of proteins. What is the function of each of the following SDS-PAGE reagents? (a) acrylamide; (b) *N,N'*-methylene bisacrylamide; (c) ammonium persulfate; (d) *N,N,N',N'*-tetramethylethylenediamine; (e) sodium dodecyl sulfate; (f) 2-mercaptoethanol; (g) buffer; (h) Coomassie Blue R-250; (i) bromophenol blue.

50.3 Test your knowledge of 'blotting' terminology. What is Western blotting, and how does it differ from Northern and Southern blotting?

50.4 Determine the M_r of subunits of a protein by SDS-PAGE. A number of proteins of known molecular weight were analysed by SDS-PAGE and the results are

shown in the table. In the same experiment, a sample of purified lactate dehydrogenase (LDH) was treated in identical fashion and run, producing a band with a relative mobility of 0.77. (a) What is the M_r of the polypeptide produced from the LDH sample?

Relative mobilities of molecular weight standards by SDS-PAGE.

Protein	M_r	Relative mobility
Myoglobin	17 200	1.00
Carbonic anhydrase	29 000	0.84
Ovalbumin	43 000	0.67
Human albumin	68 000	0.51
Transferrin	77 000	0.46

(b) If the M_r of native LDH is 136 000, what do you conclude about the subunit structure of this enzyme?

50.5 Find out about 'troubleshooting' problems with gels. Investigate the pitfalls of working with and polymerising gels.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

51 Advanced electrophoretic techniques

Using electrophoresis in proteomics – note that many of the techniques described in this chapter are employed to generate the information used in bioinformatic analysis (Chapter 70).

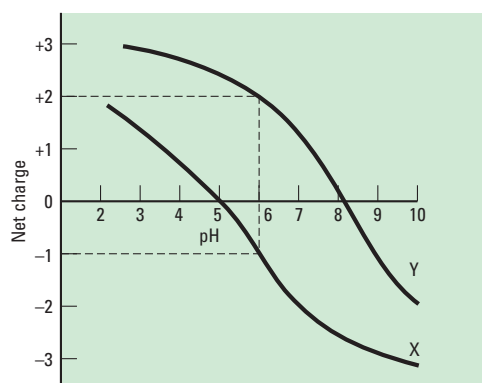


Fig. 51.1 Titration curves for two proteins, X and Y.

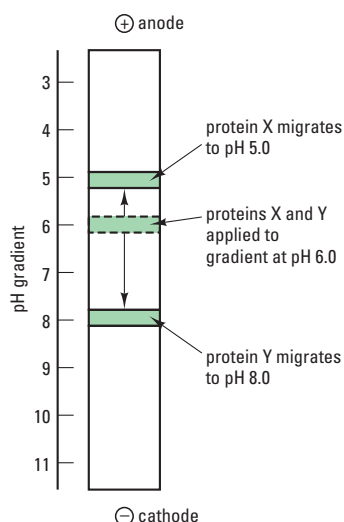


Fig. 51.2 The migration of two proteins, X and Y, in response to a pH gradient.

Although the resolution obtained using the basic electrophoretic techniques described in Chapter 50 is adequate for many applications, a number of advanced techniques are available that give very high resolution with very small amounts of sample material.

Using isoelectric focusing (IEF)

In contrast to standard electrophoresis, which is carried out at constant pH, IEF is carried out using a pH gradient. The gradient is formed using small molecular mass ampholytes, which are analogues and homologues of polyamino-, polycarboxylic acids that collectively have a range of isoelectric points (pI values) between pH 3 and 10. When a mixture of ampholytes (p. 161), either in a gel or in free solution, is placed between the anode in acid solution (for example, H_3PO_4), and the cathode in alkaline solution (for example, NaOH) and an electric field is applied, each ampholyte migrates to its own pI and forms a stable pH gradient which will persist for as long as the field is applied. When a protein sample is applied to this gradient, separation is achieved, since individual proteins will migrate to their isoelectric points. The net charge on the protein when first applied will depend on the specific 'titration curve' for that protein (Fig. 51.1). As an example, consider two proteins, X and Y, having pI values of pH 5 and pH 8 respectively, which are placed together on the gradient at pH 6 (Fig. 51.2). At that pH, protein X will have a net negative charge, and will migrate towards the anode, progressively losing charge until it reaches its pI (pH 5) and stops migrating. Protein Y will have a net positive charge at pH 6, and so will migrate towards the cathode until it reaches its pI (pH 8).

By using a polyacrylamide gel as a supporting medium and a narrow pH gradient, you can separate proteins differing in pI by as little as 0.01 units. Even greater resolution is possible in free solution (for example, in capillary electrophoresis, pp. 376–7). Such resolution is possible because protein molecules that diffuse away from the pI will acquire a net charge (negative at increased pH, positive at decreased pH) and are then immediately focused back to their pI. This focusing effect will continue for as long as the electric field is applied.

A useful variant of IEF is used to obtain *titration curves* for proteins. A pH gradient is set up, and the sample applied in a line at a right angle to the gradient. The net charge on a given protein will vary according to its position on the gradient – when electrophoresis is carried out at right angles to the pH gradient, the protein will migrate at a velocity and direction governed by that charge. When stained, each protein will appear as a continuous curved line, corresponding to its titration curve (Fig. 51.3). This technique can be useful during protein purification, prior to ion-exchange chromatography (p. 452): by obtaining the titration curve for a protein of interest and those of major contaminants, you can then select the mobile phase pH that gives optimal separation.

In IEF, it is important to avoid electro-osmotic flow (EOF, p. 366), as this would affect the ability of the proteins to remain stationary at their pIs. Use polyacrylamide to minimise EOF, in gel IEF and use narrow bore tubing with an internal polymer coating for capillary IEF.

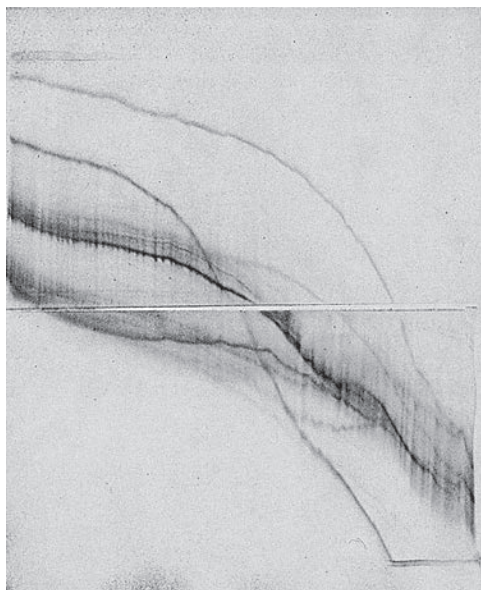


Fig. 51.3 Titration curves of bovine muscle proteins produced by electrofocusing-electrophoresis

Avoiding streaking in 2D

electrophoresis – check carefully to ensure that the sample contains no particulate material (e.g. from protein aggregation); filter or centrifuge before use.

Maximising resolution in 2D

electrophoresis of proteins – try to minimise nucleic acid contamination of your sample (see Chapter 60), as they may interact with polypeptides/proteins, affecting their movement in the gel.

Freezing gel strips – be sure to mark the identity and orientation of each gel strip before freezing, e.g. by inserting a fine wire into one end of the strip.

Carrying out two-dimensional electrophoresis

You are most likely to use this high-resolution technique to separate proteins by charge in one dimension using IEF in a polyacrylamide gel, followed by separation by molecular mass in the second dimension using denaturing SDS-PAGE (p. 368). The technique allows you to separate up to 1000 proteins from a single sample. Typically, the first dimension IEF run (pH 3–10) is carried out on gel strips of length 7–24 cm. Strips are run at a voltage of 500–3500 V for 1.5 h, then at 3500 V for a further 4 h. Following IEF, the gel strips can be used immediately, or frozen until required.

It is common for the second-dimension SDS-PAGE separation to be carried out on a discontinuous slab gel 0.5–1.5 mm thick, which includes a low %T stacking gel and a separating gel with an exponential gradient of 10–16%T. The separating gel can be prepared in advance, but the stacking gel must be formed shortly before addition of the gel from the one-dimensional run.

After equilibration with the buffer used in SDS-PAGE, load the IEF gel strip onto the 2D gel (still between the glass plates in which it was formed) and seal in position using acrylamide or agarose. Before the sealing gel sets, form a well at one end to allow addition of molecular mass markers. Run the second-dimension at 100–200 V until the tracking dye front is ≈ 1 cm from the bottom edge of the slab. After running, process the gel to detect polypeptides, for example, using Coomassie Blue or a silver stain. Analysis of the complex patterns that result from 2D electrophoresis requires computer-controlled gel scanners to acquire and process data from a gel image, such as that shown in Fig. 51.4. These systems can compare, adjust and match up patterns from several gels, allowing both accurate identification of spots and quantification of individual proteins. Allowance is made for the slight variations in patterns found in different runs, using internal references ('landmarks'), which are either added standard proteins or particular spots known to be present in all samples.

Using capillary electrophoresis (CE)

This technique combines the high resolving power of electrophoresis with the speed and versatility of HPLC (p. 349). The technique largely overcomes the major problem of carrying out electrophoresis without a supporting medium, i.e. poor resolution caused by convection currents and diffusion. A capillary tube has a high surface area : volume ratio, and consequently the heat generated as a result of the applied electric current is rapidly dissipated. A further advantage is that very small sample volumes (5–10 nl) can be analysed. The versatility of CE is demonstrated by its use in the separation of a range of biomolecules, for example, amino acids, proteins, nucleic acids, drugs, vitamins, organic acids and inorganic ions; CE can even separate neutral species, for example, steroids, aromatic hydrocarbons (see Volpi and Maccari, 2013).

The components of a typical CE apparatus are shown in Fig. 51.5. The capillary is made of fused silica and externally coated with a polymer for mechanical strength. The internal diameter is usually 25–50 μm , a compromise between efficient heat dissipation and the need for a light path that is not too short for detection using UV/visible spectrophotometry. A gap in the polymer coating provides a window for detection purposes.

Samples can be injected into the capillary by a variety of means, for example, electrophoretic loading or displacement. In the former, the inlet

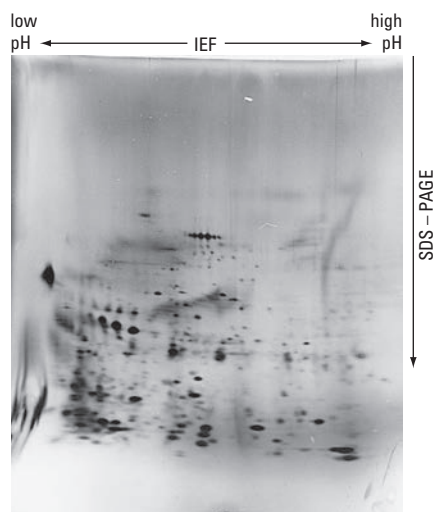


Fig. 51.4 Two-dimensional separation of proteins from 100x concentrated urine (2.5 μg total protein; silver stain).

Courtesy of T. Marshall and K.M. Williams.

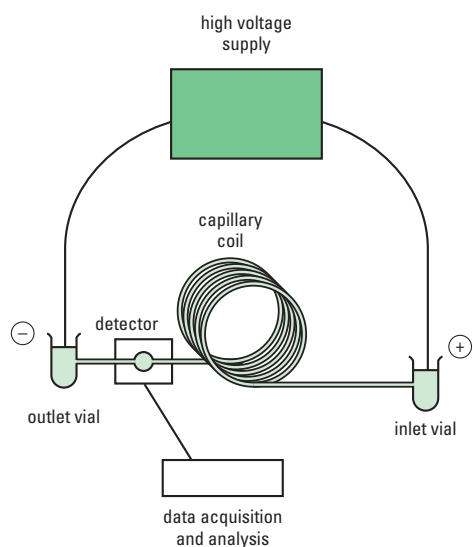


Fig. 51.5 Components of a capillary electrophoresis system.

end of the capillary is immersed in the sample and a pulse of high voltage is applied. The displacement method involves forcing the sample into the capillary, either by applying pressure in the sample vial using an inert gas, or by introducing a vacuum at the outlet. The detectors used in CE are similar to those used in chromatography, typically UV/visible spectrophotometric systems. Fluorescence detection is more sensitive, but this often requires sample derivatisation. Electrochemical and conductivity detection are used in some applications, for example, conductivity detection of inorganic cations such as Na^+ and K^+ .

Electro-osmotic flow (EOF), described on p. 366, is essential to the most commonly used types of CE. The existence of EOF in the capillary is the result of the net negative charge on the fused silica surface at pH values over 3.0. The resulting solvent flow towards the cathode is greater than the attraction of anions towards the anode, so they will flow towards the cathode (the detector is situated at the cathodic end of the capillary). The greater the net negative charge on an anion, the greater is its resistance to the EOF and the lower its mobility. Separated components migrate towards the cathode in the order: (1) cations, (2) neutral species, (3) anions.

Capillary zone electrophoresis (CZE)

This is the most widely used form of CE, and is based on electrophoresis in free solution and EOF, as discussed above. Separations are caused by the charge: mass ratio of the sample components, and the technique can be used for almost any type of charged molecule, and is especially useful for peptide separation and confirmation of purity.

Micellar electrokinetic chromatography (MEKC)

This technique involves the principles of both electrophoresis and chromatography. Its main strength is that it can be used for the separation of neutral molecules as well as charged ones. This is achieved by including surfactants (for example, SDS, Triton X-100) in the electrophoresis buffer at concentrations that promote the formation of spherical micelles, with a hydrophobic interior and a charged, hydrophilic surface. When an electric field is applied, these micelles will tend to migrate with or against the EOF, depending on their surface charge. Anionic surfactants like SDS are attracted by the anode, but if the pH of the buffer is high enough to ensure that the EOF is faster than the migration velocity of the micelles, the net migration is in the direction of the EOF, i.e. towards the cathode. During this migration, sample components partition between the buffer and the micelles (acting as a pseudo-stationary phase); this may involve both hydrophobic and electrostatic interactions. For neutral species it is only the partitioning effect that is involved in separation; the more hydrophobic a sample molecule, the more it will interact with the micelle, and the longer will be its migration time, since the micelle resists the EOF. The versatility of MEKC enables it to be used for separations of biomolecules as diverse as amino acids and polycyclic hydrocarbons. MEKC is also known as micellar electrokinetic capillary chromatography (MECC).

Chiral capillary electrophoresis (CCE)

Resolution of a pair of chiral enantiomers (optical isomers) represents one of the biggest challenges for separation science, because each member of the pair will have identical physicochemical properties. CE offers an

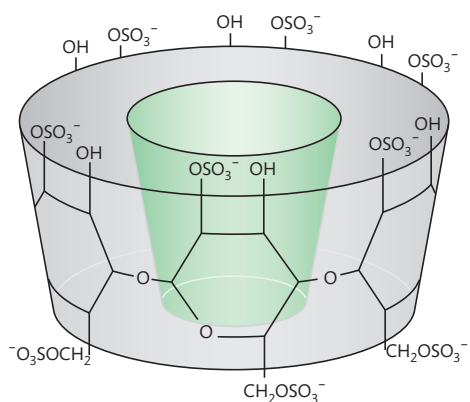


Fig. 51.6 General structure of highly sulfated cyclodextrins (HSCDs). The central cavity of the cyclodextrin (shaded green) can interact differently with the R and S isomers of a biomolecule, enabling separation in CE.

Adapted from Chiral Methods Development Kit, Beckman Coulter, Inc.

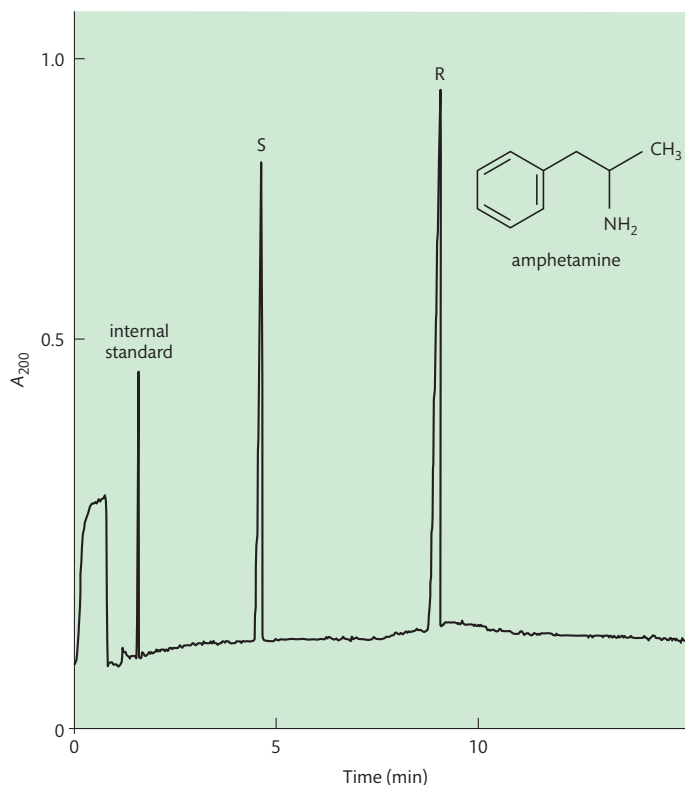


Fig. 51.7 CE separation of R and S enantiomers of amphetamine using Beckman Coulter highly sulfated gamma cyclodextrin.

Adapted from Chiral Methods Development Kit, Beckman Coulter, Inc.

Distinguishing between stereoisomers –

the R and S convention involves prioritising atoms or groups bonded to an asymmetric carbon atom in order of their atomic number. With the smallest atom or group pointing away from you, note the size of the remaining three groups or atoms. If the configuration in order of increasing size is clockwise, this is termed the R-configuration (L. *rectus*); if the order is anticlockwise, this is the S-configuration (L. *sinister*). Note that the older, alternative terminology of 'D' (L. *dextro*) and 'L' (L. *laevo*) isomers is widely used in the life sciences (p. 423), but the two terminologies do not always coincide (i.e. R is not always D, and S is not always L).

effective method of separating enantiomers by inducing a 'chiral selector' in the electrophoresis medium. The most commonly used chiral selectors are cyclodextrins such as the highly sulfated cyclodextrins (HSCDs) (Fig. 51.6). As the enantiomers migrate along the capillary, one will tend to interact more strongly than the other and its mobility will be reduced relative to the other. Figure 51.7 shows separation of the R and S forms of amphetamine using 5% HSCD in the electrophoresis buffer. The R-form has greater affinity for the HSCD used, so its retention time on the capillary is longer than that of the S-form.

Note that HSCDs can also be used in HPLC, but CCE is more effective, with shorter development times and lower reagent costs.

Capillary gel electrophoresis (CGE)

The underlying principle of this technique is directly comparable with that of conventional PAGE, i.e. the capillary contains a polymer that acts as a molecular sieve. As charged sample molecules migrate through the polymer network, larger molecules are hindered to a greater extent than smaller ones and will tend to move more slowly. CGE differs from CZE and MEKC in that the inner surface of the capillary is polymer-coated to prevent

EOF; this means that for most applications (for example, polypeptide or oligonucleotide separations) sample components will migrate towards the anode at a rate determined by their size. The technique also differs from conventional PAGE in that a ‘polymer network’ is used rather than a gel: the polymer network may be polyacrylamide or agarose.

CGE offers the following advantages over conventional electrophoresis:

- **efficient heat dissipation means that a high electrical field can be applied,** giving shorter separation times
- **detection of the separated components as they move towards the anodic end of the capillary (for example, using a UV/visible detector) means that staining is unnecessary**
- **automation is feasible.**

Choosing a detector for capillary electrophoresis

– most types of HPLC detector are suitable for CE and related applications (see Chapter 49).

Capillary isoelectric focusing (CIEF)

This is used mainly for protein separation. Here, the principles of IEF are valid as long as EOF is prevented by using capillaries that are polymer-coated on their inner surface. Sample components migrate to their isoelectric points and become stationary. Once separated ([10 min]), the components must be mobilised so that they flow past the detector. This is achieved by changing the NaOH solution in the cathodic reservoir to an NaOH/NaCl solution. When the electric field is reapplied, Cl^- ions enter the capillary, causing a decrease in pH at the cathodic end and the subsequent migration of sample components.

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[Covers the principles underlying IEF.]

STUDY EXERCISES

51.1 Consider the requirements for sample application in PAGE and IEF. Explain why in PAGE the sample is applied in a discrete narrow band, usually at the cathodic end of the gel, while in IEF the sample can be applied at any point along the length of the gel without concern about location or narrowness of the sample zone.

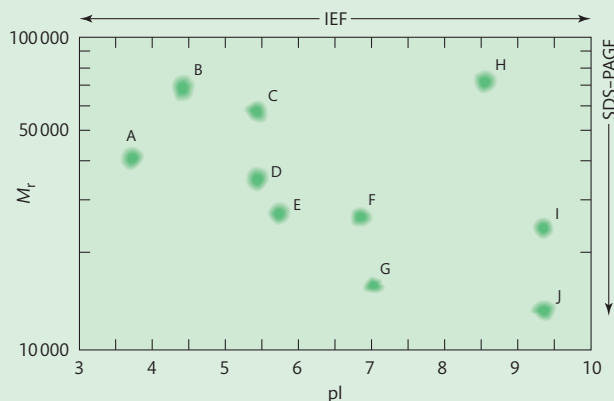
51.2 Choose a suitable CE technique for a range of applications. Which variant of CE would you consider most suitable for the following applications? (a) Separation of small peptides of similar size; (b) separation of oligonucleotides of different lengths; (c) separation of proteins with different isoelectric points; (d) a sample of urine tested for several anabolic steroids.

51.3 Check the similarities and differences between electrophoresis and isoelectric focusing. Complete the following table, indicating (i) whether each of the factors listed is relevant to each technique and (ii) briefly explain why, in each case.

Potential factors influencing electrophoresis (E) and isoelectric focusing (IEF).

Factor	E	IEF
Dependence upon ionisable groups in proteins		
Requirement for the application of an electric field		
Dependence upon differences in net charge between sample molecules		
Dependence upon differences in pI values between sample molecules		
Requirement for buffer at constant pH		
Requirement for a pH gradient		
Requirement for ampholytes to be included in solution		
Use of supporting medium with molecular sieving		
Treatment of samples with SDS is possible		
Diffusion of separated proteins is significant		

51.4 Identify proteins by 2D electrophoresis. The figure represents the separation of a mixture of 10 proteins by 2D electrophoresis. Using the information in the table, identify the individual proteins A–J.



M_r and pI values of selected protein subunits.

Protein	Subunit M_r	Subunit pI
Nerve growth factor	13 300	9.3
Ribonuclease	13 700	7.8
Haemoglobin	16 000	7.0
β -lactoglobulin	17 500	5.2
Ceramide trihexosidase	22 000	3.0
Trypsinogen	24 500	9.3
Triose phosphate isomerase	27 000	5.7
Galactokinase	27 000	5.7
Arginase	30 000	9.2
Glycerol-3-phosphate dehydrogenase	34 000	6.4
Alcohol dehydrogenase	35 000	5.4
Deoxyribonuclease II	38 000	10.2
Aldolase	40 000	5.2
Pepsinogen	41 000	3.7
Hexokinase	51 000	5.3
Lipoxidase	54 000	5.7
Catalase	57 500	5.4
Alkaline phosphatase	69 000	4.4
Acetylcholine esterase	70 000	4.5
Glyceraldehyde-3-phosphate dehydrogenase	72 000	8.5

Answers to these study exercises are available at go.pearson.com/uk/he/resources

52 Electroanalytical techniques

Definitions

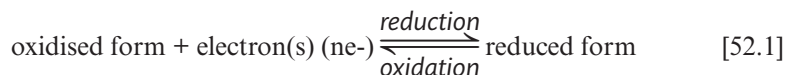
Oxidation – loss of electrons by an atom or molecule (or gain of O atoms, loss of H atoms, increase in positive charge, or decrease in negative charge).

Reduction – gain of electrons by an atom or molecule (or loss of O atoms, gain of H atoms, decrease in positive charge or increase in negative charge).

Electrochemical methods are used to quantify a broad range of different biomolecules, including ions, gases, metabolites, drugs and hormones. They make use of changes in electrical potential (potentiometry), current (voltammetry) or charge (coulometry) to determine the concentration of a biomolecule in solution. These methods often provide a cost-effective and rapid means of assay, as described below.

KEY POINT The basis of all electrochemical analysis is the transfer of electrons from one atom or molecule to another atom or molecule in an obligately coupled oxidation–reduction reaction (a redox reaction).

When considering electrochemical redox reactions, it is convenient to consider them in terms of the two half-reactions by convention, written as:



You should note that each half-reaction is reversible: by applying suitable conditions, reduction *or* oxidation can take place. As an example, a simple redox reaction occurs when metallic zinc (Zn) is placed in a solution containing copper ions (Cu^{2+}), as follows:



The half-reactions are (i) $\text{Cu}^{2+} + 2\text{e}^- \rightarrow \text{Cu}$ and (ii) $\text{Zn}^{2+} + 2\text{e}^- \rightarrow \text{Zn}$. The oxidising power of (i) is greater than that of (ii), so in a coupled system the latter half-reaction proceeds in the opposite direction to that shown above, i.e. as $\text{Zn} - 2\text{e}^- \rightarrow \text{Zn}^{2+}$. When Zn and Cu electrodes are placed in separate solutions containing their ions, and connected electrically (Fig. 52.1), electrons will flow from the Zn electrode to Cu^{2+} via the Cu electrode owing to the difference in oxidising power of the two half-reactions.

By convention, the electrode potential of any half-reaction is expressed relative to that of a standard hydrogen electrode (half-reaction $2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2$) and is called the standard electrode potential, E° . Table 52.1 shows the values of E° for selected half-reactions. With any pair of half-reactions from this series, electrons will flow from that having the lowest electrode potential to that of the highest. E° is determined at $\text{pH} = 0$. It is often more appropriate to express standard electrode potentials at $\text{pH} 7$ for biological systems, and the symbol E°' is used: in all circumstances, it is important that the pH is clearly stated.

The arrangement shown in Fig. 52.1 represents a simple galvanic cell where two electrodes serve as the interfaces between a chemical system and an electrical system. For analytical purposes, the magnitude of the potential (voltage) or the current produced by an electrochemical cell is related to the concentration (strictly, the activity, a , pp. 152–3) of a particular chemical species. Electrochemical methods offer the following advantages:

- **excellent detection limits**, and wide operating range (10^{-1} – 10^{-8} mol L^{-1})
- **measurements may be made on very small volumes** (μL), allowing small amounts (pmol) of sample to be measured in some cases

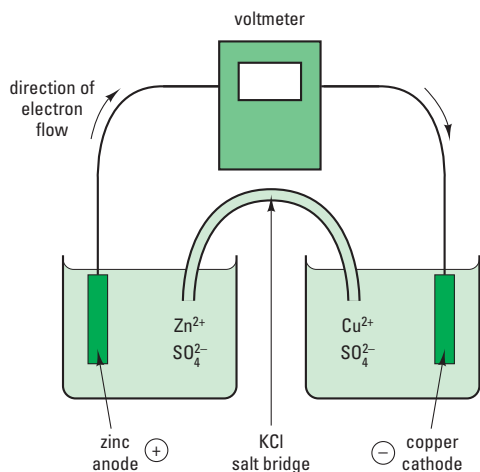


Fig. 52.1 A simple galvanic electrochemical cell. The KCl salt bridge allows migration of ions between the two compartments but prevents mixing of the two solutions.

Definition

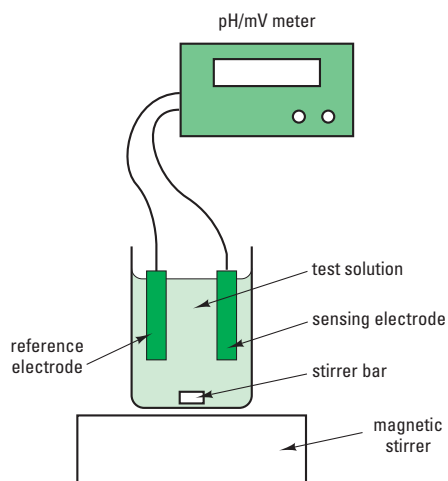
Galvanic cell – an electrochemical cell in which reactions occur spontaneously at the electrodes when they are connected externally by a conductor, producing electrical energy.

Table 52.1 Standard electrode potentials* (E°) for selected half-reactions.

Half-reaction	E° at 25° C (V)
$\text{Cl}_2 + 2\text{e}^- \rightleftharpoons 2\text{Cl}^-$	+1.36
$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightleftharpoons 2\text{H}_2\text{O}$	+1.23
$\text{Br}_2 + 2\text{e}^- \rightleftharpoons 2\text{Br}^-$	+1.09
$\text{Ag}^+ + \text{e}^- \rightleftharpoons \text{Ag}$	+0.80
$\text{Fe}^{3+} + \text{e}^- \rightleftharpoons \text{Fe}^{2+}$	+0.77
$\text{I}_3^- + 2\text{e}^- \rightleftharpoons 3\text{I}^-$	+0.54
$\text{Cu}^{2+} + 2\text{e}^- \rightleftharpoons \text{Cu}$	+0.34
$\text{Hg}_2\text{Cl}_2 + 2\text{e}^- \rightleftharpoons 2\text{Hg} + 2\text{Cl}^-$	+0.27
$\text{AgCl} + \text{e}^- \rightleftharpoons \text{Ag} + \text{Cl}^-$	+0.22
$\text{Ag}(\text{S}_2\text{O}_3)_2^{3-} + \text{e}^- \rightleftharpoons \text{Ag} + 2\text{S}_2\text{O}_3^{2-}$	+0.01
$2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{H}_2$	+0.00
$\text{AgI} + \text{e}^- \rightleftharpoons \text{Ag} + \text{I}^-$	-0.15
$\text{PbSO}_4 + 2\text{e}^- \rightleftharpoons \text{Pb} + \text{SO}_4^{2-}$	-0.35
$\text{Cd}^{2+} + 2\text{e}^- \rightleftharpoons \text{Cd}$	-0.40
$\text{Zn}^{2+} + 2\text{e}^- \rightleftharpoons \text{Zn}$	-0.76

*From Milazzo *et al.* (1978). Reproduced with permission of John Wiley & Sons Ltd.

Using a calomel electrode – before making a measurement, always ensure that the KCl solution is saturated by checking that KCl crystals are present.

**Fig. 52.2** Components of a potentiometric cell.

- miniature electrochemical sensors can be used for certain *in vivo* measurements, for example pH, glucose, oxygen content.

Using potentiometric systems and ion-selective electrodes

These systems involve galvanic cells (p. 381) and are based on measurement of the electrical potential (voltage) difference between two electrodes in solution when no net current flows between them: no net electrochemical reaction occurs and measurements are made under equilibrium conditions. These systems include methods for measuring pH (Chapter 24), ions and gases such as CO_2 and NH_3 . A typical potentiometric cell is shown in Fig. 52.2. It contains two electrodes:

1. A **'sensing' electrode**, the half-cell potential of which responds to changes in the activity (concentration) of the substance to be measured; the most common type of indicator electrodes are ion-selective electrodes (ISE).
2. A **'reference' electrode**, the potential of which does not change, forming the second half of the cell.

To assay a particular analyte, the potential difference between these electrodes is measured by a millivolt meter (for example, a standard pH meter).

Reference electrodes for potentiometry are of three main types:

1. **The standard hydrogen electrode**, which is the reference half-cell electrode, defined as 0.0 V at all temperatures, against which values of E° are expressed. H_2 gas at 1 atmosphere pressure is bubbled over a platinum electrode immersed in an acid solution with an activity of unity. This electrode is rarely used for analytical work, since it is unstable and other reference electrodes are easier to construct and use.
2. **The calomel electrode** (Fig. 52.3), which consists of a paste of mercury covered by a coat of calomel (Hg_2Cl_2), immersed in a saturated solution of KCl. The half-reaction: $\text{Hg}_2\text{Cl}_2 + 2\text{e}^- \rightarrow 2\text{Hg} + 2\text{Cl}^-$ gives a stable standard electrode potential of +0.24 V.
3. **The silver/silver chloride electrode**. This is a silver wire coated with AgCl and immersed in a solution of constant chloride concentration. The half-reaction: $\text{AgCl} + \text{e}^- \rightarrow \text{Ag} + \text{Cl}^-$ gives a stable, standard electrode potential of +0.20 V.

KEY POINT Ion-selective electrodes (ISEs) are based on measurement of a potential across a membrane which is selective for a particular analyte.

An ISE consists of a membrane, an internal electrode and an internal standard electrolyte of fixed activity. The ISE is immersed in a sample solution that contains the analyte of interest, along with a reference electrode, creating a potentiometric cell (Fig. 52.2). The membrane is chosen to have a specific affinity for a particular ion, and if the activity of this ion in the sample differs from that in the reference electrolyte, a potential develops across the membrane that is dependent on the ratio of these activities. Since the potentials of the two reference electrodes (internal

and external) are fixed, and the internal electrolyte is of constant activity, the measured potential, E , is dependent on the membrane potential and is given by the Nernst equation (Robinson and Stokes, 2002) as:

$$E = K + 2.303 \frac{RT}{zF} \log[a] \quad [52.3]$$

Using ISEs, including pH electrodes – standards and samples *must* be measured at the same temperature, as the Nernst equation shows that the measured potential is temperature-dependent.

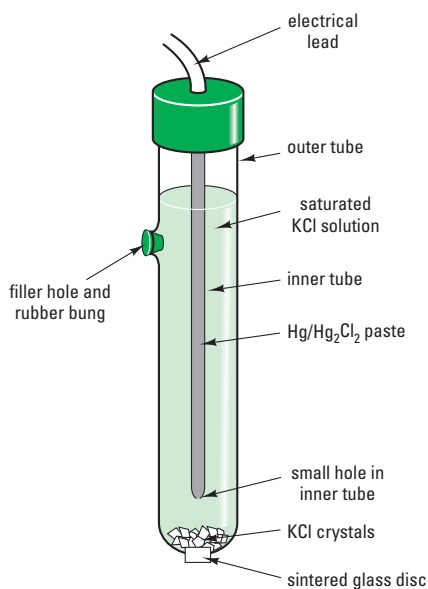


Fig. 52.3 A calomel reference electrode.

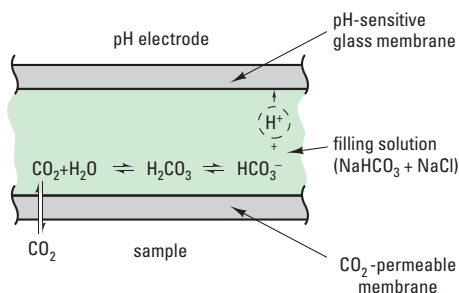


Fig. 52.4 Underlying principles of a gas-sensing electrode.

where K represents a constant potential which is dependent on the reference electrode, z represents the net charge on the analyte, $[a]$ represents the activity of analyte in the sample and all other symbols and constants have their usual meaning. For a series of standards of known activity, a plot of E against $\log[a]$ should be linear over the working range of the electrode, with a slope of $2.303 RT/zF$ (0.059 V at 25 °C). Although ISEs strictly measure *activity*, the potential differences can be approximated to concentration as long as (i) the analyte is in dilute solution, (ii) the ionic strength of the calibration standards matches that of the sample, for example by adding appropriate amounts of a high ionic strength solution to the standards, and (iii) the effect of binding to sample macromolecules (for example, proteins, nucleic acids) is minimal. Potentiometric measurements on undiluted biological fluids, for example K^+ and Na^+ levels in plasma, tissue fluids or urine, are likely to give lower values than flame emission spectrophotometry, since the latter procedure measures total ion levels, rather than just those in aqueous solution.

All of the various types of membrane used in ISEs operate by incorporating the ion to be analysed into the membrane, with the accompanying establishment of a membrane potential. The scope of electrochemical analysis has been extended to measuring gases and non-ionic compounds by combining ISEs with gas-permeable membranes, enzymes and even immobilised bacteria or tissues.

Glass membrane electrodes

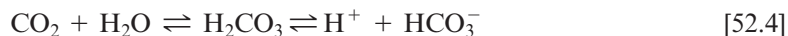
The most widely used ISE is the glass membrane electrode for pH measurement (p. 161). The membrane is composed of thin glass (50 μm wall thickness) made of silica which contains some Na^+ . When the membrane is soaked in water, a thin hydrated layer is formed on the surface in which negative oxide groups ($Si-O^-$) in the glass act as ion exchange sites. If the electrode is placed in an acid solution, H^+ exchanges with Na^+ in the hydrated layer, producing an external surface potential: in alkaline solution, H^+ moves out of the membrane in exchange for Na^+ . Since the inner surface potential is kept constant by exposure to a fixed activity of H^+ , a consistent, accurate potentiometric response is observed over a wide pH range. Glass electrodes for other cations (for example, Na^+ , NH_4^+) have been developed by changing the composition of the glass, so that it is predominantly sensitive to the particular analyte, though the specificity of such electrodes is not absolute. The operating principles and maintenance of such electrodes are broadly similar to those for pH electrodes (p. 161).

Gas-sensing glass electrodes

Here, an ISE in contact with a thin external layer of aqueous electrolyte (the 'filling solution') is kept close to the glass membrane by an additional, outer membrane that is selectively permeable to the gas of interest. The arrangement for a CO_2 electrode is shown in Fig. 52.4: in this case the outer membrane is made of CO_2 -permeable silicone rubber. When CO_2 gas in the

Using a CO₂ electrodes – applications include measurement of blood $p\text{CO}_2$ and in enzyme studies where CO₂ is utilised or released: calibration of the electrode is accomplished using 5%v/v and 10%v/v mixtures of CO₂ in an inert gas equilibrated against the measuring solution.

sample selectively diffuses across the membrane and dissolves in the filling solution (in this case an aqueous NaHCO₃/NaCl mixture), a change in pH occurs owing to the shift in the equilibrium:



The pH change is detected by the internal ion-selective pH electrode, and its response is proportional to the partial pressure of CO₂ of the solution ($p\text{CO}_2$). A similar principle operates in the NH₃ electrode, where a Teflon membrane is used, and the filling solution is NH₄Cl.

Liquid and polymer membrane electrodes

In this type of ISE, the liquid is a water-insoluble viscous solvent containing a soluble ionophore, i.e. an organic ion-exchanger, or a neutral carrier molecule, that is specific for the analyte of interest. When this liquid is soaked into a thin membrane such as cellulose acetate, it becomes immobilised. The arrangement of analyte (A^+) and ionophore in relation to this membrane is shown in Fig. 52.5. The potential on the inner surface of the membrane is kept constant by maintaining a constant activity of A^+ in the internal solution, so the potential change measured is that which results from A^+ in the sample interacting with the ionophore in the outer surface of the membrane.

A relevant example of a suitable ionophore is the antibiotic valinomycin (p. 475), which specifically binds K^+ . Other ionophores have been developed for measurement of, for example, NH_4^+ , Ca^{2+} , Cl^- . In addition, electrodes have been developed for organic species by using specific ion-pairing reagents in the membrane that interact with ionic forms of the organic compound, for example, with drugs such as 5,5-diphenylhydantoin.

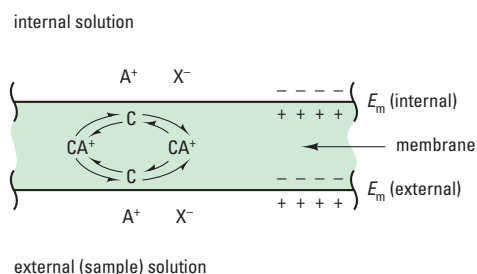


Fig. 52.5 Underlying principles of a liquid membrane ion-selective electrode. A^+ = analyte; C = neutral carrier ionophore; E_m = surface potential; membrane potential = $E_m(\text{internal}) - E_m(\text{external})$.

Definition

Ionophore – a compound that enhances membrane permeability to a specific ion: an ionophore may be incorporated into an ISE as a means of detecting that ion.

Definitions

Electrolysis – a non-spontaneous chemical reaction resulting from the application of a potential to an electrode.

Electrolytic cell – an electrochemical cell in which reactions are driven by the application of an external voltage greater than the spontaneous potential of the cell.

Solid-state membrane electrodes

These contain membranes made from single crystals or pressed pellets of salts of the analyte. The membrane material must show some permeability to ions and must be virtually insoluble in water. Examples include:

- the fluoride electrode, which uses LaF_3 impregnated with Eu^{2+} (the latter to increase permeability to F^-). A membrane potential is set up when F^- in the sample solution enters spaces in the crystal lattice
- the chloride electrode, which uses a pressed-pellet membrane of Ag_2S and AgCl .

Voltammetric methods

These are based on measurements made using an electrochemical cell in which electrolysis is occurring. Voltammetry, sometimes also called amperometry, involves the use of a potential applied between two electrodes (the working electrode and the reference electrode) to cause oxidation or reduction of an electroactive analyte. The loss or gain of electrons at an electrode surface causes current to flow, and the size of the current (usually measured in mA or μA) is directly proportional to the concentration of the electroactive analyte. The materials used for the working electrode must be good conductors and electrochemically inert, so that they simply transfer

Applying oxygen electrode technology – the system developed by Leyland C. Clark is used in the Rank Brothers oxygen electrode, forming the basis of many practical exercises across the biosciences.

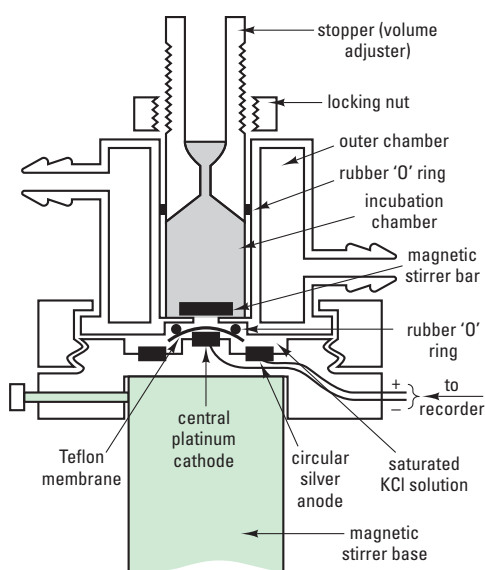


Fig. 52.6 Transverse section through a Clark (Rank) oxygen electrode.

Using polytetrafluoroethylene (PTFE) membranes – these are readily permeable to many small molecules and are used for monitoring of dissolved gases in aqueous systems.

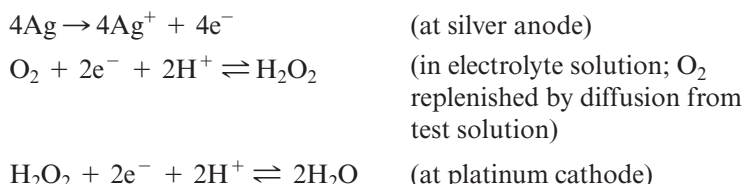
electrons to and from species in solution. Suitable materials include Pt, Au, Hg and glassy carbon.

Two widely used devices that operate on the voltammetric principle are the oxygen electrode and the glucose electrode. These are sometimes referred to as amperometric sensors.

Oxygen electrodes

The Clark (Rank) oxygen electrode

These instruments measure oxygen in solution using the polarographic principle, i.e. by monitoring the current flowing between two electrodes when a voltage is applied. The most widespread electrode is the Clark cell type (Fig. 52.6), manufactured by Rank Bros, Cambridge, UK, which is suitable for measuring O_2 concentrations in cell, organelle and enzyme suspensions. Pt and Au electrodes are in contact with a solution of electrolyte (normally saturated KCl). The electrodes are separated from the medium by a Teflon membrane, permeable to O_2 . When a potential is applied across the electrodes, this generates a current proportional to the O_2 concentration (Hoffman and Clokie, 2018). The reactions can be summed up as:



Setting up and using a Clark (Rank) oxygen electrode

Box 52.1 describes the steps involved: if you are setting up from scratch, perform steps 1–13; if a satisfactory membrane is already in place, start at step 7. Box 52.2 gives advice on how to use a data logger or chart recorder with an oxygen electrode, including how to calculate rates of oxygen consumption/production, using data for oxygen saturation of air-equilibrated water shown in Table 52.2.

The temperature of the incubation vessel should be controlled by passing water (for example from a water bath) through the outer chamber (Fig. 52.6). Cells or organelles may be present in the solution added to the incubation chamber or can be added via the hole in the stopper using a syringe, as can chemicals such as inhibitors (Box 52.2). Take care not to introduce air bubbles, and remove any that appear by *gently* raising and lowering the stopper. The electrode can be used repeatedly, providing the membrane is satisfactory: remove solutions carefully (for example using a pipette, or vacuum line and trap). Keep water in the chamber when not in use. Replace the membrane if:

- the reading becomes noisy
- the electrode will not zero after adding sodium dithionite
- the response becomes too slow (check by switching off the in-built stirrer – oxygen concentration should drop rapidly as the available.

KEY POINT Successful operation of a Clark (Rank) oxygen electrode requires an intact Teflon membrane and clean electrodes. When replacing the Teflon membrane, you should always check that the electrode surfaces are clean (shiny); if necessary, use a mild abrasive paste to remove any oxidised material.

Box 52.1 How to set up a Clark (Rank) oxygen electrode

- 1. Detach the base of the incubation vessel** (see Fig. 54.6) by unscrewing the locking ring.
- 2. Add enough saturated KCl to cover the electrodes.**
- 3. Cut a 1-mm square hole in the centre of a 10 × 10-mm square of lens tissue** and place this on the KCl solution so that the hole is over the central platinum cathode.
- 4. Cut a 10 × 10-mm square of Teflon membrane and place over the lens tissue;** seal by gently lowering the incubation vessel and tightening the locking ring, making sure that the rubber O-ring is correctly positioned over the membrane.
 - (a) Do not overtighten the locking ring.
 - (b) Take care not to trap air bubbles beneath the membrane.
 - (c) Make sure that the membrane does not become twisted.
- 5. Clamp the electrode over the magnetic stirrer base using the clamping screw.**
- 6. Connect the electrode leads to the polarising unit/recording device** (silver anode to positive, platinum cathode to negative). You can either use a digital readout system on the control unit or the output can be passed to a chart recorder, giving a readout of oxygen status as a function of time (see Box 52.2). Check that the polarising voltage is set to 0.60 V and adjust, if necessary, using the 'polarising voltage' control (use a small screwdriver to adjust).
- 7. Add air-saturated experimental solution and a small Teflon-coated magnetic stirrer bar to the chamber.** The volume of the incubation chamber can be adjusted by moving the locking nut on the stopper. To adjust, add the appropriate volume of liquid to the chamber using a pipette, insert the stirrer bar and stopper and screw the locking nut on the stopper until the solution just fills the incubation chamber.
- 8. Gently push the stopper (volume adjuster) into position,** making sure that no air bubbles are trapped in the chamber, and switch on the stirrer. Adjust the rate of stirring using the 'stirrer' control, if required.
- 9. Set the zero:** remove the stopper and add a few crystals of sodium dithionite – the dithionite ions ($\text{S}_2\text{O}_4^{2-}$) will be oxidised to sulfite (SO_3^{2-}) and sulfate ions ($\text{S}_2\text{O}_4^{2-}$), thereby consuming all of the oxygen in the solution within 3–5 min (an alternative is to bubble N_2 through the solution for >10 min). Once the reading has stabilised, if this is not at zero, then adjust using the 'set zero' control (in practice, the zero is usually quite stable and often needs no adjustment).
- 10. Adjust the sensitivity:** rinse out the dithionite solution thoroughly/repeatedly, then replace with air-equilibrated water and allow the reading to stabilise (this may take 5–10 min.). Then adjust the 'sensitivity' control of the electrode system to set the oxygen saturated value at an appropriate point (for a controller with a digital readout, this is best set to 100, and then the values represent percentage oxygen saturation). To check that the instrument is working correctly, switch off the stirrer for a few moments – the reading should drop quickly as oxygen is consumed at the electrode surface and should then rise rapidly when the stirrer is switched back on.
- 11. Rinse the incubation chamber thoroughly and add fresh experimental solution.** Make sure that all traces of sodium dithionite are removed.
- 12. Carry out your experiment.**
- 13. Remove the solution and check the calibration.** If the reading for air-equilibrated water is different, the electrode's sensitivity or the temperature may have changed and you may need to recalibrate and repeat the measurement.

Box 52.2 How to convert a chart recorder trace to a rate of O₂ consumption or production

Having set up your oxygen electrode (Box 54.1) or probe, the most common experiments are those in which you measure the rate of oxygen consumption (respiration) or production (photosynthesis) in a fixed volume of solution (e.g. in the chamber of a Rank (Clark) oxygen electrode). This may involve using a data logger and carrying out subsequent numerical and graphical analysis using a spreadsheet (Chapter 72). However, in undergraduate lab classes it may also involve attaching the electrode to a chart recorder and following the change in oxygen concentration with time. The principal steps are as follows.

- 1. Calculate the amount of oxygen in the electrode chamber** – multiply the appropriate oxygen-saturation concentration, from Table 52.2, by the volume of the chamber. For example, if you are working in mmol L^{-1} ($= \mu\text{mol mL}^{-1}$) then at 20°C, air-equilibrated (oxygen-saturated) distilled water contains $0.285 \mu\text{mol mL}^{-1}$. For an electrode chamber of volume 5 mL, there will be an amount of $0.285 \times 5 = 1.425 \mu\text{mol}$ oxygen in the water within the chamber at saturation.
- 2. Set the zero on the recorder** – following the addition of a small amount of sodium dithionite to the chamber, to remove oxygen (Box 52.1), the trace is monitored until it has stabilised. Then adjust the 'zero' or 'back off' control of the recorder until the chart trace is set to zero on the chart paper: this is marked as 'A' on Fig. 52.8.
- 3. Set the oxygen-saturated reading on the recorder** – rinse out the dithionite solution thoroughly/repeatedly, replace with air-equilibrated water and allow the reading to stabilise (this may take 3–5 min). Then adjust the 'sensitivity' control of the chart recorder to set the oxygen-saturated value at an appropriate point on the chart – for respiration measurements, this can be close to the full width of the chart paper, whereas for photosynthesis measurements it is more usual to set it closer to the mid-point of the chart paper, to allow measurements over 100% to be recorded. In the example shown in Fig. 52.8, where the chart paper is calibrated in millimetre divisions, the oxygen-saturated reading is set at 80 divisions (80 mm), marked as 'B'. Note that the recorder pen can be lifted between readings, and during rinsing of the chamber, to reduce the amount of 'noise' on the chart.
- 4. Calculate the amount of oxygen equivalent to a single division of the chart paper** – divide the amount of oxygen

in the chamber by the number of divisions (mm) to give the amount per division (e.g. $1.425 \div 80 = 0.0178125 \mu\text{mol}$ ($= 17.8125 \text{ nmol per division}$)).

- 5. Carry out your experiment** – the example shown in Fig. 52.8 represents the consumption of oxygen during respiration of a suspension of yeast cells, marked as 'C'. Continue the recording until you are satisfied that the rate has remained stable for at least 5 min. In many experiments, you will be adding substances that change the rate (e.g. metabolic inhibitors, or substrates) – this can be done by injecting a solution containing the substance through the small hole in the stopper of the electrode (keep the volume of this solution to $<1\%$ of the total volume of the chamber, to minimise the effect of the added solution).
- 6. Convert the gradient of the trace into a rate of oxygen consumed or produced** – using a transparent ruler, draw the line of best fit through that part of the trace showing a stable relationship (see Fig. 52.8). Use this line of best fit to work out the gradient of the trace, by converting from linear dimensions to an amount per unit time; e.g. the gradient shown in Fig. 52.8 is equivalent to a decrease of 24 chart divisions (mm) in 3 cm. Using the value calculated in step 4, the amount of oxygen consumed is thus $17.8125 \times 24 = 427.5 \text{ nmol}$. For a chart speed of 0.2 cm min^{-1} , this represents the change occurring over a time interval of $3 \div 0.2 = 15 \text{ min}$. Thus the rate of oxygen consumed is $427.5 \div 15 = 28.5 \text{ nmol min}^{-1}$.
- 7. Express the rate on an appropriate basis** – in most instances, this will be either per cell (e.g. determined using a haemocytometer, p. 245) or per unit mass of protein (p. 426), chlorophyll or an equivalent marker. For the example shown in Fig. 52.8, if there were 2.4×10^7 yeast cells per mL of suspension, then in the 5-mL chamber of the electrode there would be a total of $2.4 \times 10^7 \times 5 = 1.2 \times 10^8$ yeast cells, giving a rate of $28.5 \div (1.2 \times 10^8) = 2.375 \times 10^{-7} \text{ nmol min}^{-1} \text{ cell}^{-1}$, probably better expressed as $237.5 \text{ amol min}^{-1} \text{ cell}^{-1}$. Note that when inhibitors/ substrates are used, you should always wait until a new stable rate has been achieved (for around 5 min) before using the trace to determine the new rate of oxygen consumption/ production.

Table 52.2 Oxygen saturation values for distilled water and sea water at standard atmospheric pressure and a range of temperatures (derived from Green and Carritt, 1967).

Temperature (°C)	Distilled water			Sea water		
	mol m ⁻³ (mmol L ⁻¹)	mg L ⁻¹ (ppm)	ml L ⁻¹	mol m ⁻³ (mmol L ⁻¹)	mg L ⁻¹ (ppm)	ml L ⁻¹
0	0.460	14.7	10.3	0.359	11.5	8.04
2	0.435	13.9	9.75	0.342	10.9	7.65
4	0.413	13.2	9.24	0.326	10.4	7.30
6	0.392	12.5	8.78	0.311	9.95	6.97
8	0.373	11.9	8.35	0.298	9.54	6.66
10	0.355	11.4	7.95	0.285	9.12	6.37
12	0.339	10.8	7.59	0.273	8.74	6.11
14	0.324	10.4	7.25	0.261	8.35	5.85
16	0.310	9.92	6.94	0.251	8.03	5.62
18	0.297	9.50	6.65	0.241	7.71	5.40
20	0.285	9.12	6.38	0.232	7.42	5.19
22	0.274	8.77	6.12	0.224	7.17	5.00
24	0.263	8.42	5.89	0.215	6.88	4.82
26	0.253	8.1	5.67	0.208	6.66	4.64
28	0.244	7.81	5.46	0.200	6.40	4.48
30	0.235	7.52	5.27	0.193	6.18	4.32
37	0.211	6.75	4.71	0.174	5.57	3.88

Notes: mol m⁻³ ≡ mmol L⁻¹ ≡ μmol mL⁻¹ ≡ nmol μL⁻¹. Tabulated values assume atmospheric pressure = 101.3 kPa (/760 mmHg); for more accurate work, a correction for any deviation can be made by multiplying the appropriate figures from the table by the ratio of the real pressure to the assumed pressure.

Definition

Biosensor – a device for measuring a substance, combining the selectivity of a biological reaction with that of a sensing electrode.

Oxygen probes

Clark-type oxygen electrodes are also available in probe form for immersion in the test solution (Fig. 52.7), for example for field studies, allowing direct measurement of oxygen status *in situ*, in contrast to chemical assays.

KEY POINT Note that the solution must be stirred during oxygen measurement, to replenish the oxygen consumed by the electrode, owing to the 'boundary layer' effect: gentle movement of the probe during measurement provides sufficient agitation.

Enzymatic glucose electrodes

These are simple types of biosensor; a simple design is shown in Fig. 52.9. It consists of a Pt electrode, overlaid by two membranes. Sandwiched between these membranes is a layer of the immobilised enzyme glucose oxidase. The outer membrane is glucose-permeable and allows glucose in the sample to diffuse through to the glucose oxidase layer, where it is converted to gluconic acid and H₂O₂. The inner membrane is selectively permeable to H₂O₂, which is oxidised to O₂ at the surface of the Pt electrode. The current arising from this release of electrons is proportional to the glucose

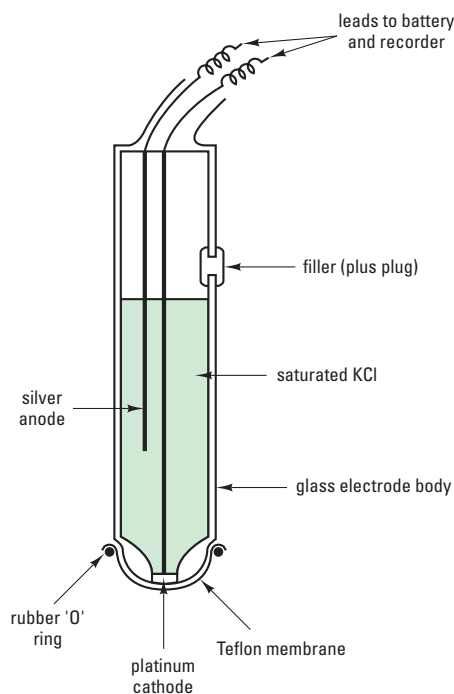


Fig. 52.7 A Clark-type oxygen probe.

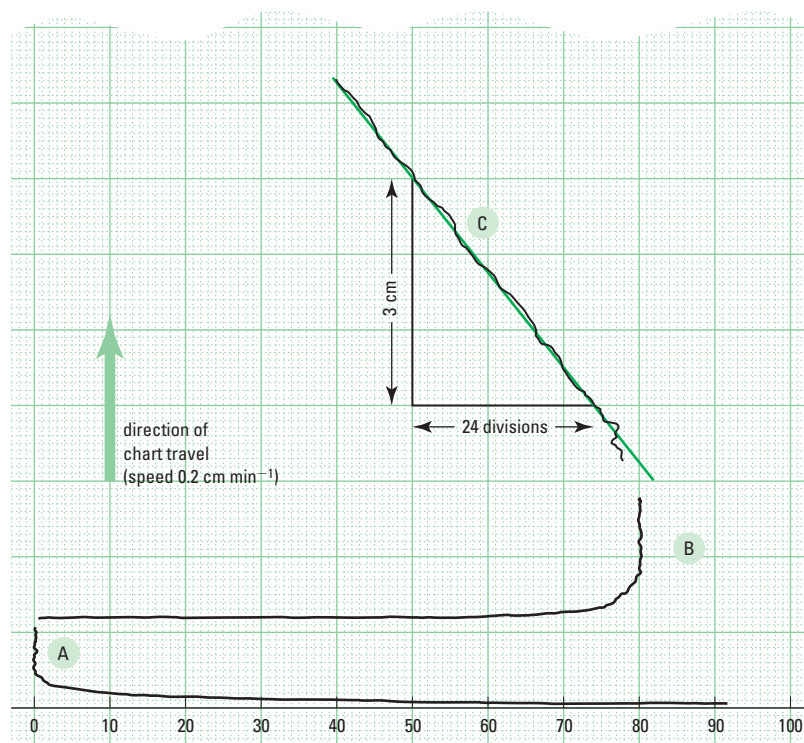


Fig. 52.8 Chart recorder output for a Clark (Rank) oxygen electrode. A = zero oxygen; B = oxygen saturation; C = respiration of yeast.

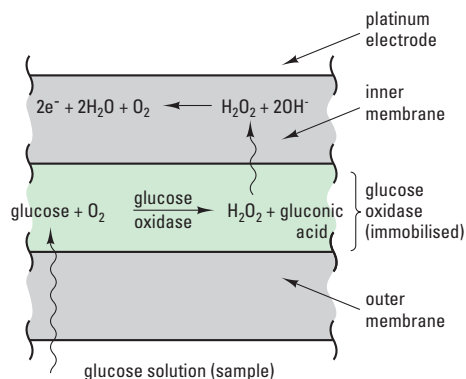


Fig. 52.9 Underlying principles of a glucose electrode.

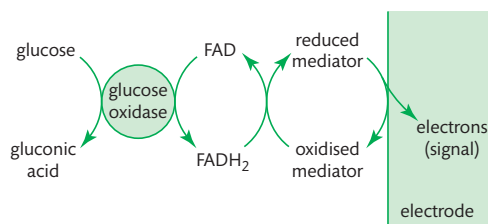


Fig. 52.10 Operating principles of a glucose biosensor. In the ExacTech system, the mediator is a ferrocene derivative.

concentration in the sample within the range 10^{-7} – 10^{-3} mol L⁻¹. The efficiency of a biosensor can be increased by including an electrode surface that is capable of capturing electrons directly. This is the basis of personal blood glucose meters for use by diabetic people, for example the ExacTech meter (Abbott Laboratories). Here a pinprick blood sample is applied to a test strip containing glucose oxidase; if glucose is present, a series of reactions occurs as shown in Fig. 52.10.

The electrons produced are donated to the electrode surface, producing an initial current that is proportional to the initial rate at which glucose is oxidised which, in turn, is proportional to the glucose concentration in the blood sample. The future design of biosensors is likely to involve microchip technology in which the biocatalyst and the transducer are even more intimately linked.

Electrochemical detectors used in chromatography operate by voltammetric principles and currents are produced as the mobile phase flows over electrodes set at a fixed potential: to achieve maximum sensitivity, this potential must be set at a level that allows electrochemical reactions to occur in all analytes of interest.

Applying coulometric methods

Here, the *charge* required to electrolyse a sample completely is measured: the time required to titrate an analyte is measured at constant current and related to the amount of analyte using Faraday's law. There are few biological applications of this technique, though it is sometimes used for determination of Cl⁻ in serum and body fluids.

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Sources for further study

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[Homepage of the manufacturing company, Rank Brothers – contains technical manuals and contact information.]

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STUDY EXERCISES

52.1 Test your understanding of the circumstances under which ion-selective electrodes can be used. List the three main assumptions underlying the measurement of ion concentration using an ion-selective electrode.

52.2 Test your knowledge of biosensor design. List the two main functional components of a biosensor such as the glucose electrode. Research a specific application – note that it should be different from the ones used in this chapter (for example, using the web).

52.3 Calculate the oxygen content of specified volumes of water at defined temperatures. Using the information in Table 54.2 and assuming air equilibration at 101.3 kPa, what is the amount of oxygen in each of the following (give all answers to three significant figures):

- (a) 4 mL of distilled water at 20 °C (express your answer in μmol);
- (b) 20 mL of sea water at 12 °C (express your answer in μmol);

- (c) 10 mL of distilled water at 15 °C (express your answer in μmol);
- (d) 250 mL of distilled water at 37 °C (express your answer in mg);
- (e) 200 mL of sea water at 25 °C (express your answer in mL).

52.4 Calculate respiration using data from an oxygen electrode readout. An oxygen electrode was set up to contain 5.0 mL of air-equilibrated distilled water at 30 °C, giving a full-scale reading on a chart recorder (200 divisions on the chart recorder paper), while a zero reading gave no deflection (0 divisions on the chart recorder paper). A yeast cell suspension (containing 4.1×10^9 cells in total) was added to the same electrode system along with a suitable growth medium, giving an approximately linear trace on the chart recorder with a slope of -25 units per cm, at a chart speed of 0.5 cm min^{-1} .

(continued)

STUDY EXERCISES (continued)

What is the respiratory oxygen consumption in $\text{nmol min}^{-1} (10^9 \text{ cells})^{-1}$? (Give your answer to three significant figures.)

52.5 Determine net photosynthetic rate from oxygen electrode readings. The data below represent oxygen electrode readings taken every minute from 0 min for an illuminated suspension of a cyanobacterium in a 5.0-mL chamber of an oxygen electrode at 20 °C: 70.4, 73.6, 75.3, 78.5, 83.6, 85.9, 88.2, 91.4, 94.1. The system

was calibrated to read 100.0 for air-equilibrated distilled water and 0.0 for anaerobic water, in effect making the scale read in terms of percentage oxygen saturation. The chlorophyll *a* content of an equivalent amount of cyanobacterial suspension to that used in the electrode chamber was measured at $2.13 \mu\text{g mL}^{-1}$. What is the net photosynthetic rate of the cyanobacterial suspension, in $\mu\text{mol min}^{-1} (\text{mg chlorophyll } a)^{-1}$? (Express your answer to three significant figures.)

Answers to these study exercises are available at go.pearson.com/uk/he/resources

53 Using immunological methods

Definitions

Antibody – a protein produced in response to an antigen (an *antibody-generating* foreign macromolecule).

Epitope – a site on the antigen that determines its interaction with a particular antibody.

Hapten – a substance that contains at least one epitope, but is too small to induce antibody formation unless it is linked to a macromolecule.

Ligand – a molecule or chemical group that binds to a particular site on another molecule.

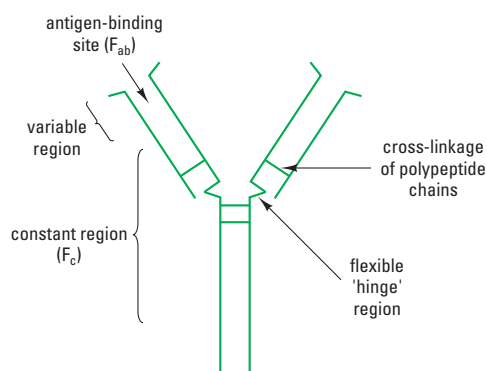


Fig. 53.1 Diagrammatic representation of IgG (antibody).

Producing polyclonal antibodies – in the UK, this is controlled by government regulations, since it involves vertebrate animals: personnel must be licensed by the Home Office and must operate in accordance with the Animals Scientific Procedures Act (1986) and with the Code of Practice for the Housing and Care of Animals (2005).

Antibodies are an important component of the immune system, which protects animals against certain diseases (see Delves *et al.*, 2017). They are produced by B lymphocytes in response to foreign macromolecules (antigens). A particular antibody will bind to a site on a specific antigen, forming an antigen–antibody complex (immune complex). Immunological bioassays use the specificity of this interaction for:

- **identifying macromolecules, cellular components or whole cells**
- **quantifying a particular substance.**

Understanding antibody structure

An antibody is a complex globular protein, or immunoglobulin (Ig). Although there are several types, IgG is the major soluble antibody in vertebrates and is used in most immunological assays. Its main features are:

- **shape:** IgG is a Y-shaped molecule (Fig. 53.1), with two antigen-binding sites.
- **specificity:** variation in amino acid composition at the antigen-binding sites explains the specificity of the antigen–antibody interaction.
- **flexibility:** each IgG molecule can interact with epitopes that are different distances apart, including those on different antigen molecules.
- **labelling:** regions other than the antigen-binding sites can be labelled, for example, using a radioisotope or an enzyme with fluorogenic or chromogenic detection.

KEY POINT The presence of two antigen-binding sites on a single flexible antibody molecule is relevant to many immunological assays, especially the agglutination and precipitation reactions.

Producing analytical antibodies

Polyclonal antibodies

These are commonly used at undergraduate level. They are produced by repeated injection of antigen into a laboratory animal. After a suitable period (3–4 weeks) blood is removed and allowed to clot, leaving a liquid phase (polyclonal antiserum) containing many different IgG antibodies, resulting in:

- **cross-reaction** with other antigens or haptens
- **batch variation**, as individual animals produce slightly different antibodies in response to the same antigen
- **non-specificity**, as the antiserum will contain many other antibodies.

Standardisation of polyclonal antisera therefore is difficult. You may need to assess the amount of cross-reaction, inter-batch variation or non-specific binding using appropriate controls, assayed at the same time as the test samples.

Definition

Monoclonal antibody – an antibody produced by a laboratory-grown clone of either a hybridoma (a fusion of an antibody-producing lymphocyte and a myeloma or lymphoma cell) or a virus-transformed lymphocyte.

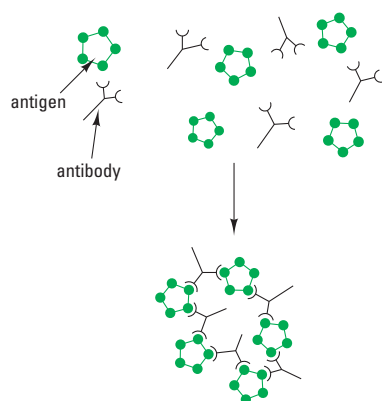


Fig. 53.2 Formation of an antigen-antibody complex (immune complex).

Monoclonal antibodies

These are specific to a single epitope and are produced from individual clones of cells (hybridomas, p. 296), grown using cell culture techniques (p. 296). Such cultures provide a stable source of antibodies of known, uniform specificity. Although monoclonal antibodies are likely to be used increasingly in future years, polyclonal antisera are currently employed for many routine immunological assays.

Carrying out agglutination tests

When antibodies interact with a suspension of a particulate antigen, for example, cells or latex particles, the formation of immune complexes (Fig. 53.2) causes visible clumping, termed agglutination. A positive haemagglutination reaction gives an even 'carpet' of red cells over the base of the tube, while a negative reaction gives a tightly packed 'button' of red cells at the bottom of the tube. Agglutination tests are used in several ways:

- **Microbial identification:** at the species or subspecies level (serotyping), for example, mixing an unknown bacterium with the appropriate antiserum will cause the cells to agglutinate.
- **Latex agglutination using bound antigens:** by coating soluble (non-particulate) antigens onto microscopic latex spheres, their reaction with a particular antibody can be visualised.
- **Latex agglutination using bound antibodies:** antibodies can be bound to latex microspheres, leaving their antigen-binding sites free to react with soluble antigen.
- **Haemagglutination:** red blood cells can be used as agglutinating particles. However, in some instances, such reactions do not involve antibody interactions (for example, some animal viruses may haemagglutinate unmodified red blood cells).

Carrying out precipitin tests

Immune complexes of antibodies and soluble antigens (or haptens) usually settle out of solution as a visible precipitate: this is termed a precipitin test, or precipitation test. The formation of visible immune complexes in agglutination and precipitation reactions only occurs if antibody and antigen are present in an optimal ratio (Fig. 53.3). It is important to appreciate the shape of this curve: cross-linkage is maximal in the zone of equivalence, decreasing if either component is present in excess. Visual assessment of precipitation reactions forms the basis of several other techniques, described below.

Carrying out immunodiffusion assays

These techniques are easier to perform and interpret than the quantitative precipitin test. Precipitation of antibody and antigen occurs within an agarose gel, giving a visible line corresponding to the zone of equivalence (Fig. 53.3). Details of the main techniques are given in Box 53.1.

Performing immunoelectrophoretic assays

These methods combine the precipitin reaction with electrophoretic migration, providing sensitive, rapid assays with increased separation and resolution.

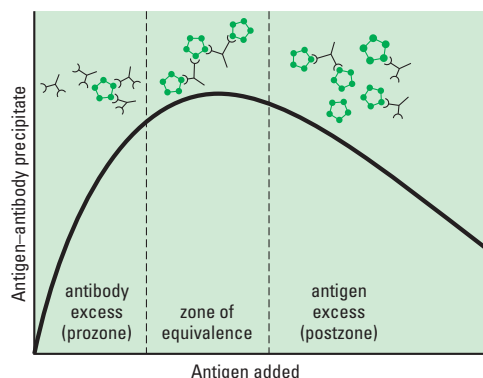


Fig. 53.3 Precipitation curve for an antigen titrated against a fixed amount of antibody.

Box 53.1 How to carry out immunodiffusion assays

The two most widespread approaches are (i) single radial immunodiffusion (Mancini technique) and (ii) double diffusion immunoassay (Ouchterlony technique).

Single radial immunodiffusion (RID) (Mancini technique)

This is used to quantify the amount of antigen in a test solution, as follows:

1. **Prepare an agarose gel** (1.5% w/v) containing a fixed amount of antibody: allow to set on a glass slide or plate, on a level surface.
2. **Cut several circular wells in the gel.** These should be of a fixed size between 2 and 4 mm in diameter (see Fig. 53.4(a)). Cut your wells carefully – they should have straight sides and the agarose must not be torn or lifted from the glass plate. All wells should be filled to the top, with a flat meniscus, to ensure identical diffusion characteristics. Non-circular precipitin rings, resulting from poor technique, should not be included in your analysis.
3. **Add a known amount of the antigen or test solution to each well.**
4. **Incubate on a level surface at room temperature in a moist chamber:** diffusion of antigen into the gel produces a precipitin ring. This is usually measured after 2–7 days, depending on the molecular mass of the antigen.
5. **Examine the plates against a black background** (with side illumination), or stain using a protein dye (e.g. Coomassie blue).

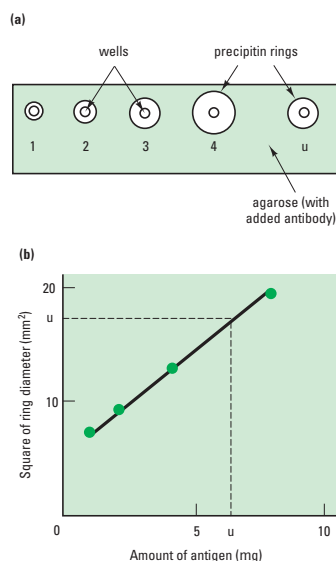


Fig. 53.4 Single radial immunodiffusion (RID). (a) Assay: four standards are shown (wells 1 to 4, each one double the strength of the previous standard), and an unknown (μ), run at the same time. (b) Calibration curve. The unknown contains 6.25 μg of antigen. Note the non-zero intercept of the calibration curve, corresponding to the square of the well diameter: do not force such calibration lines through the origin.

6. **Measure the diameter of the precipitin ring**, e.g. using Vernier callipers.
7. **Prepare a calibration curve** (Chapter 43) from the samples containing known amounts of antigen (Fig. 53.4(b)): the squared diameter of the precipitin ring is directly proportional to the amount of antigen in the well.
8. **Quantify the amount of antigen in your test solutions** using a calibration curve prepared from standards assayed at the same time.

Double diffusion immunoassay (Ouchterlony technique)

This technique is widely used to detect particular antigens in a test solution, or to look for cross-reaction between different antigens.

1. **Prepare an agarose gel** (1.5% w/v) on a level glass slide or plate: allow to set.
2. **Cut several circular wells in the gel.**
3. **Add test solutions of antigen or polyclonal antiserum to adjacent wells.** Both solutions diffuse outwards, forming visible precipitin lines where antigen and corresponding antibody are present in optimal ratio (Fig. 53.5).

The various reactions between antigen and antiserum are:

- **Identity:** two wells containing the same antigen, or antigens with identical epitopes, will give a fused precipitin line (identical interaction between the antiserum and the test antigens, Fig. 53.5(a)).
- **Non-identity:** where the antiserum contains antibodies to two different antigens, each with its own distinct epitopes, giving two precipitin lines which intersect without any interaction (no cross-reaction, Fig. 53.5(b)).
- **Partial identity:** where two antigens have at least one epitope in common, but where other epitopes are present, giving a fused precipitin line with a spur (cross-reaction, Fig. 53.5(c)).

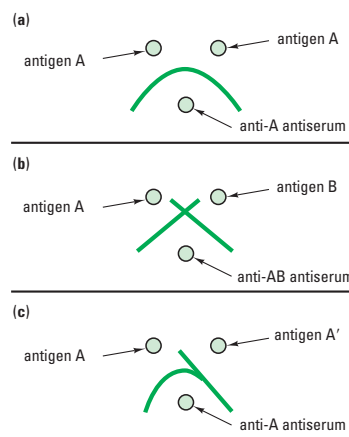


Fig. 53.5 Precipitin reactions in double diffusion immunoassay: (a) identity; (b) non-identity; (c) partial identity.

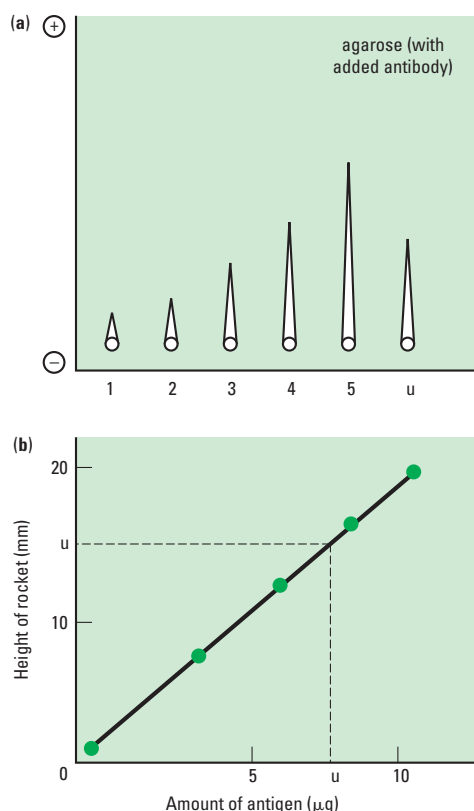


Fig. 53.6 Laurell rocket immunoelectrophoresis. (a) Assay: precipitin rockets are formed by electrophoresis of five standards of increasing concentration (wells 1 to 5) and an unknown (μ). (b) Calibration curve: the unknown sample contains 7.7 μ g of antigen.

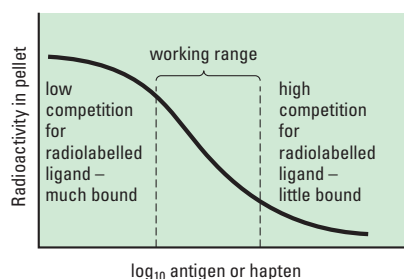


Fig. 53.7 Radioimmunoassay calibration curve. Note that the assay is insensitive at very low and very high antigen levels.

Cross-over electrophoresis (counter-current electrophoresis)

This is similar to the Ouchterlony technique, since antigen and antibody are in separate wells. However, the movement of antigen and antibody towards each other is driven by a voltage gradient (pp. 364–5): most antigens migrate towards the anode, while IgG migrates towards the cathode. This method is faster and more sensitive than double immunodiffusion, taking 15–20 min to reach completion.

Quantitative immunoelectrophoresis (Laurell rocket immunoelectrophoresis)

This is similar to RID, as the antibody is incorporated into an agarose plate while the antigen is placed in a well. However, a voltage gradient moves the antigen into the gel, usually towards the anode, while the antibody moves towards the cathode, giving a sharply peaked, rocket-shaped precipitin line once equivalence is reached (within 2–10 h). The height of each rocket shape at equivalence is directly proportional to the amount of antigen added to each well. A calibration curve for samples containing a known amount of antigen can be used to quantify the amount of antigen present in test samples (Fig. 53.6).

Using radioimmunological methods

These methods use radioisotopes to detect and quantify the antigen–antibody interaction, giving improved sensitivity over agglutination and precipitation methods.

Radioimmunoassay (RIA)

This is based on competition between a radioactively labelled antigen (or hapten) and an unlabelled antigen for the binding sites on a limited amount of antibody. The quantity of antigen in a test solution can be determined using a known amount of radiolabelled antigen and a fixed amount of antibody (Fig. 53.7). As with other immunoassay methods, it is important to perform appropriate controls to screen for potentially interfering compounds. The basic procedure for RIA is as follows:

- 1. Add appropriate volumes of a sample to a series of small test tubes.**
Prepare a further set of tubes containing known quantities of the substance to be assayed to provide a standard curve.
- 2. Add a known amount of radiolabelled antigen (or hapten) to each tube** (sample and standard).
- 3. Add a fixed amount of antibody to each tube** (the antibody must be present in limited quantity).
- 4. Leave at constant temperature for a fixed time** (usually 24 h), to allow antigen–antibody complexes to form.
- 5. Precipitate the antibody and bound antigen using saturated ammonium sulfate, followed by centrifugation.**
- 6. Determine the radioactivity of the supernatant or the precipitate** (pp. 393–5).
- 7. Prepare a calibration curve of radioactivity against \log_{10} antigen** (Fig. 53.7). The curve is most accurate in the central region, so adjust the amount of antigen in your test sample to fall within this range.

Note the following:

- **You must be registered to work with radioactivity:** check health and safety and other requirements (p. 409) with the Departmental Safety Officer.
- **Measure all volumes as accurately as possible** as the end result depends on the volumetric quantities of unlabelled (sample) antigen, radio-labelled antigen and antibody: an error in any of these reagents will invalidate the assay.
- **Incorporate replicates**, so that errors can be quantified.
- **Seek your supervisor's advice** about fitting a curve to your data: this can be a complex process.

Immunoradiometric assay (IRMA)

This technique uses radiolabelled antibody, rather than antigen, for direct measurement of the amount of antigen (or hapten) in a sample. Most IRMAs are similar to the double antibody sandwich method described below, except that the second antibody is labelled using a radioisotope. The important advantages over RIA are:

- **linear relationship between amount of radioactivity and test antigen**
- **wider working range for test substance**
- **improved stability/longer shelf-life.**

Performing enzyme immunoassays (EIA)

These techniques are also known as enzyme-linked immunosorbent assays (ELISA). They combine the specificity of the antibody–antigen interaction with the sensitivity of enzyme assays using either an antibody or an antigen conjugated (linked) to an enzyme at a site that does not affect the activity of either component. The enzyme is measured by adding an appropriate chromogenic substrate (p. 461), which yields a coloured product. Enzymes offer the following advantages over radioisotopic labels:

- **increased sensitivity:** a single enzyme molecule can produce many product molecules, amplifying the signal.
- **simplified assay:** enzyme assays are usually easier than radioisotope assays (p. 460).
- **improved stability of reagents:** components are generally more stable than their radiolabelled counterparts, giving them a longer shelf-life.
- **no radiological hazard:** no requirement for specialised containment/disposal facilities.
- **automation is straightforward:** using disposable microtitre plates and an optical scanner.

The principal techniques are double-antibody-sandwich ELISA, indirect ELISA and competitive ELISA.

Double-antibody-sandwich ELISA

This is used to detect specific antigens, involving a three-component complex between a capture antibody linked to a solid support, the antigen, and a second, enzyme-linked antibody (Fig. 53.8). This can be used to detect a particular antigen, for example, a virus in a clinical sample, or to quantify the amount of antigen.

Using chromogenic substrates for enzyme immunoassay – these include tetramethylbenzidine (Box 53.2), which is a highly sensitive substrate used to detect horseradish peroxidase activity, producing a blue-coloured product as a result of peroxidase activity.

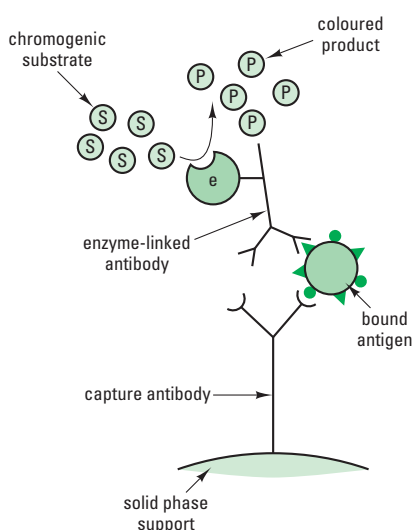


Fig. 53.8 Double antibody sandwich ELISA.

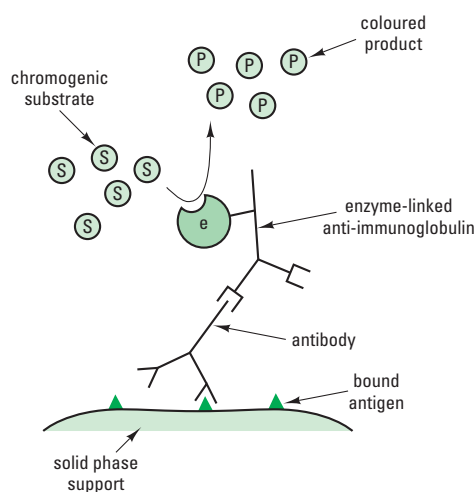


Fig. 53.9 Indirect ELISA.

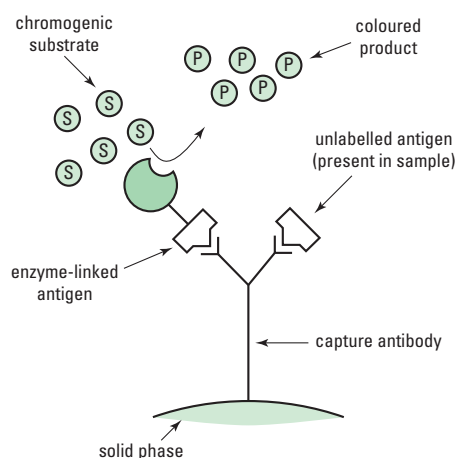


Fig. 53.10 Competitive ELISA.

Understanding lateral flow testing – the UK's rapid lateral flow test for SARS-CoV-2 (coronavirus) operates on the same chromatographic principle as a dipstick immunoassay (Fig. 53.11), except that the strip is kept in a horizontal position and a small amount of sample is added to one end, travelling across the strip by capillary action.

Indirect ELISA

This is used for antibody detection, with a specific antigen attached to a solid support. When the appropriate antibody is added, it binds to the antigen and will not be washed away during rinsing. Bound antibody is then detected using an enzyme-linked anti-immunoglobulin, for example, a rabbit IgG antibody raised against human IgG (Fig. 53.9). One advantage of the indirect assay is that a single enzyme-linked anti-immunoglobulin can be used to detect several different antibodies, since the specificity is provided by the bound antigen.

Competitive ELISA

Here, any antigen present in a test sample competes with added enzyme-labelled antigen for a limited number of binding sites on the capture antibody (Fig. 53.10). Most commercial systems use 96-well microplates (12 columns by 8 rows), where each well is coated with the appropriate antibody. Following addition of known volumes of (i) sample and (ii) enzyme-labelled antigen, the plates are incubated (typically, up to 1 h), then washed thoroughly to remove all unbound material. Bound enzyme-labelled antigen is then detected using a suitable substrate. Quantitative results can be obtained by measuring the absorbance of each well using a spectrophotometric microplate reader: the absorbance at a particular wavelength is *inversely* proportional to the amount of antigen present in the test sample. Calibration standards (p. 314) are required to convert the readings to an amount or concentration. Alternatively, the test can be carried out in positive/negative format. Box 53.2 gives practical details for a sandwich-type ELISA.

Using dipstick (chromatographic) immunoassays

Improvements in technology have led to the development of a range of diagnostic kits that can be used either in a medical setting ('near-patient testing') or in the home, including immunoassays for cancer screening and drug testing. The first of these available to the general public was the home pregnancy test kit which measures the hormone human chorionic gonadotrophin (hCG) in urine. The level of this hormone is raised substantially during pregnancy. The principle of this test, which is similar to other home test kits, is that coloured plastic microbeads are conjugated to an anti-hCG monoclonal antibody and then coated onto an absorbent plastic or cellulose strip. When this strip is dipped into urine, any hCG present will bind to the anti-hCG antibody and both hormone-antibody complexes and free antibody will then move up the strip by capillary action until they reach a second hCG-specific antibody that is coupled chemically to the strip so that it cannot move. Any hormone-antibody complex will bind to this 'fixed' antibody (through the hCG component); owing to the presence of the coloured plastic microbeads, a distinct line will then appear at this location on the strip, signifying the presence of the hormone. If no hormone-antibody complex is present, the first antibody will simply continue up the strip until it reaches an antibody raised against the anti-hCG antibody itself, coupled to the strip at a higher point. This will bind any free anti-hCG antibody in the sample and a coloured line will appear at this point. Since there will always be an excess of the original anti-hCG antibody conjugated to the plastic beads, the appearance of two coloured

Box 53.2 How to perform an ELISA assay

While the following example is for a sandwich (capture) ELISA assay in microplate format, the same general principles apply to the other types of ELISA:

1. Prepare the apparatus. Switch on all equipment required:

- (a) The microplate reader – used to measure the absorbance of the solution in each well: set the reader to the required wavelength.
- (b) The microplate washer (where used) – each well must be washed at various stages during the procedure. When using an automated washer, first check that the wash bottle contains sufficient diluent and then test using an old microplate, to check that all wells are being washed correctly. Where required, use a wire needle to clean any blocked wash delivery tubes and repeat. For manual washing, use a wash bottle or multi-channel pipettor – make sure you fill each well and empty out all of the wash solution at the end of each wash stage.
- (c) The computer – this will contain the software required to label the wells, draw the calibration curve and calculate the results for test samples: fill out the ELISA template with details of the assays to be carried out.

2. Prepare the various solutions to be analysed. These include:

- (a) Test samples – make sure that each sample is identified with a code that enables you to record what each test well contains.
- (b) Calibrators/standards – including ‘cutoff’ calibrators and known positive standards.
- (c) Controls – positive and negative controls and blanks.

3. Coat the wells with the capture antibody. Typically 100 μL of a solution of the appropriate monoclonal or polyclonal antibody is added to each well and microplates are then incubated overnight at 4 °C to allow binding to the well.

4. Wash the wells. Transfer the microplate to the washer (or wash manually) – wash six times to remove excess coating (capture) antibody. The final rinse should be programmed so that the washer leaves the wells empty of diluent.

5. Add blocking solution to each well. Typically 100 μL of an inert protein solution (bovine serum albumin) is

added to each well to block any free binding sites on the well. Microplates are incubated at room temperature for 30 min and then washed, as in step 4.

6. Add test samples, calibrators and controls to wells.

Typically 100 μL of appropriately diluted test sample, control, etc. is added to each well. Microplates are incubated at room temperature for 90 min and then washed as in step 4.

7. Add the detection antibody to each well. Add 100 μL of monoclonal or polyclonal detection antibody labelled with a suitable enzyme (e.g. horseradish peroxidase, HRP) to each well. The microplate is then incubated at room temperature for 30–60 min and then washed, as in step 4.

8. Add chromogenic substrate to each well. For example, with HRP-labelled antigen add 100 μL of a standard solution of tetramethylbenzidine (TMB) and hydrogen peroxide to each well and re-incubate in darkness for 30 min, to allow colour development. The TMB is oxidised in the presence of hydrogen peroxide to produce a blue colour.

SAFETY NOTE TMB and hydrogen peroxide are harmful by inhalation – use a fume hood.

9. Stop the reactions. For example, by adding 100 μL of 2 mol L^{-1} sulphuric acid to denature the enzyme. The colour of the oxidised TMB will change from blue to yellow as a result of the pH shift. While the human eye can readily distinguish different shades of blue, it is more difficult to visually assess different shades of yellow, once the reaction has been stopped.

10. Measure the absorbance of each sample/calibrator/control well. Transfer the microplate to the reader and assay at an appropriate wavelength: for TMB, use 450 nm.

11. Interpret the results. Check that the absorbance values of calibrators and control are within the required range. Then, for each sample, either record the absolute value (convert to concentration or amount, e.g. using a calibration curve, Chapter 43) or record as ‘positive’ or ‘negative’ (based on values for ‘cutoff’ calibrators), as appropriate.

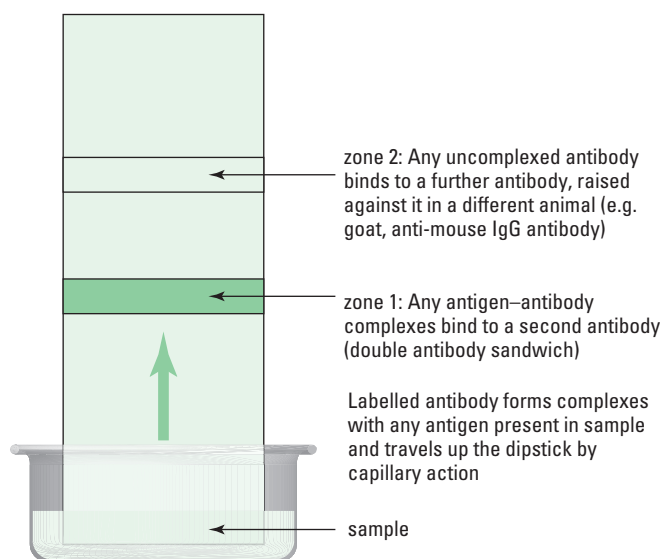


Fig. 53.11 Two-site dipstick immunoassay. For a positive test, as shown here, coloured bands will appear at zones 1 and 2 while a negative test will give colour in zone 2 only (any other colour options indicate a failed test).

lines means that the hormone is present and the test result is positive, while if only the second coloured line appears, it means that the test is negative (this also serves as a control to confirm that the test is working – lack of any coloured lines indicates that the test has failed). Figure 53.11 shows the general operating principles.

Working with complement-based assays

Understanding complement-based assays – the principal benefit from using the complement system is that it operates as an amplifying cascade of reactions, providing higher sensitivity than assays based on agglutination or precipitin reactions. For example, antibodies can be detected at concentrations of less than $1 \mu\text{g mL}^{-1}$.

Complement is a term applied to a group of around 20 different proteins which are involved in various aspects of immune function, including (i) control of inflammation, (ii) preparation of microbial cells for phagocytosis (opsonisation), (iii) lysis of target cells (for example, antibody-coated cells) and (iv) activation of leucocytes (for details see Delves *et al.*, 2011). At a practical level, the complement system can be used to detect (i) antibodies, (ii) antigens or (iii) antibody–antigen complexes, often via its lytic action on antibody-coated (‘sensitised’) cells. The operating principles of a complement fixation assay are illustrated in Fig. 53.12 for antibody detection, with the following steps:

- 1. Prepare serial doubling dilutions** (p. 155) of a test sample containing an unknown amount of antibody.
- 2. Add a standard amount of antigen:** antigen–antibody complexes will be formed in direct proportion to the amount of antibody present in the sample.
- 3. Then, add a known amount of complement to the mixture:** if immune complexes (Fig. 53.2) are present, they will ‘fix’ complement (i.e. the complement components will be consumed and no longer ‘free’ in solution), whereas if immune complexes are not present, or are at low concentration, complement will remain ‘free’ in solution, and available for subsequent reaction.

4. In the final step, add an indicator system for unbound complement – sensitised sheep red blood cells (sSRBCs) coated with rabbit anti-erythrocyte antibodies are often used. Unfixed complement is detected by lysis of the sensitised erythrocytes. The lowest dilution showing complement fixation (i.e. no erythrocyte lysis) gives the titre of antibody in the test sample.

The test can be modified to test for antigens in a sample by titration against a fixed amount of added antibody. In all cases, suitable controls are required, to check for non-specific reactions and to confirm that immune complexes are not already present in the sample, since these will fix complement and invalidate the titration. Complement fixation tests are useful for many classes of IgG and for IgM, but not for most other antibody types.

Example Complement fixation tests are the basis of the Wasserman test to detect anti-treponemal antibodies in the serum of patients with syphilis.

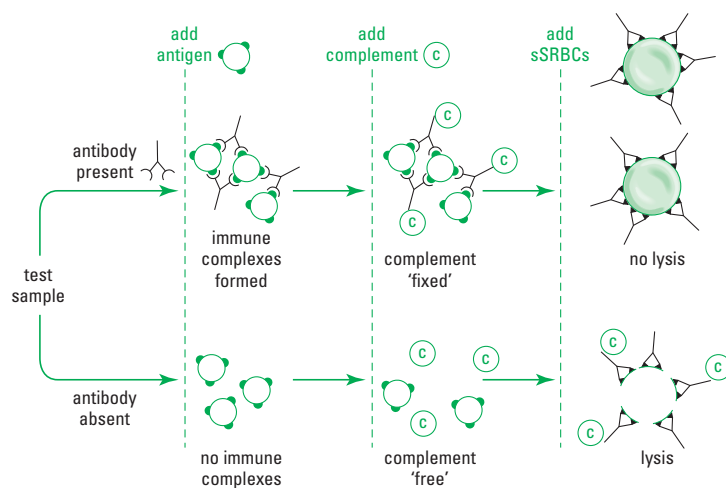


Fig. 53.12 Complement fixation test for antibody. (sSRBCs: sensitised sheep red blood cells.)

Text reference

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Sources for further study

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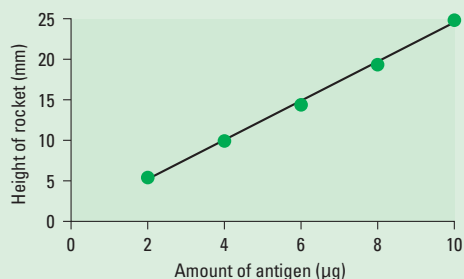
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STUDY EXERCISES

- 53.1 Determine the amount of antigen in a test solution using Laurell rocket immunoelectrophoresis.** The figure below represents the results from a rocket immunoelectrophoresis assay for a series of standards. Determine the amount of antigen in a test solution giving a rocket (precipitin line) height of 18.5 mm.



- 53.2 Determine the amount of antigen in a test solution using single radial immunodiffusion (RID).** Using the following data for a series of standards, determine the amount of antigen in a test solution giving a ring diameter of 6.5 mm.

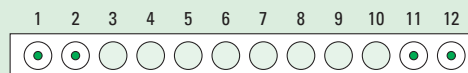
Single radial immunodiffusion results for a series of standards containing 420 µg antigen

Amount of antigen (µg)	Ring diameter (mm)
4	3.5
8	5.5
12	7.0
16	8.0
20	9.0

- 53.3 Interpret data from a haemagglutination test.**

In some conditions, such as Hashimoto's disease, autoantibodies to the thyroid protein thyroglobulin are produced in large amounts. The level of these antibodies in serum can be measured by indirect haemagglutination: red blood cells coated with thyroglobulin are mixed with serial doubling dilutions of the test serum. The titre of antithyroglobulin autoantibodies is the lowest dilution at which haemagglutination occurs. The figure below represents the outcome of an antithyroglobulin haemagglutination test on a serum sample. The first well – numbered 1 – represents a 10-fold dilution of the serum sample, with serial doubling dilutions in subsequent wells.

- (a) Write a brief explanation of the test results shown in the figure.
- (b) Determine the autoantibody titre.
- (c) Score the sample as 'positive' if the titre is more dilute than 1 in 5000 (i.e. $1 : > 5000$) and 'negative' if it is less dilute than 1 in 5000 (i.e. $1 : < 5000$).

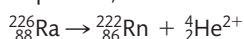


Answers to these study exercises are available at go.pearson.com/uk/he/resources

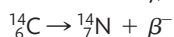
54 Using radioisotopes

Examples $^{12}_6\text{C}$, $^{13}_6\text{C}$ and $^{14}_6\text{C}$ are three of the isotopes of carbon. About 98.9% of naturally occurring carbon is in the stable $^{12}_6\text{C}$ form. $^{13}_6\text{C}$ is also a stable isotope but it only occurs at 1.1% natural abundance. Trace amounts of radioactive $^{14}_6\text{C}$ are found naturally; this is a negatron-emitting radioisotope (see Table 54.2).

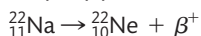
Examples $^{226}_{88}\text{Ra}$ decays to $^{222}_{86}\text{Rn}$ by loss of an alpha particle, as follows:



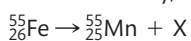
$^{14}_6\text{C}$ shows beta decay, as follows:



$^{22}_{11}\text{Na}$ decays by positron emission, as follows:



$^{55}_{26}\text{Fe}$ decays by electron capture and the production of an X-ray, as follows:



The decay of $^{22}_{11}\text{Na}$ by positron emission (β^+) leads to the production of a γ ray when the positron is annihilated on collision with an electron.

The isotopes of a particular element have the same number of protons in the nucleus but a different number of neutrons, giving them the same proton number (atomic number) but a different nucleon number (mass number, i.e. number of protons + number of neutrons). Isotopes may be stable (Chapter 55) or radioactive. Radioactive isotopes (radioisotopes) disintegrate spontaneously at random to yield radiation and a decay product.

KEY POINT Experimentation with radioisotopes is relatively specialised and advanced. It is unlikely to be used in undergraduate practicals, but it may be relevant for certain type of projects. If you do use radioactive chemicals, you will have to understand and follow relevant safety protocols.

Understanding radioactive decay

There are three forms of radioactivity (Table 54.1) arising from three main types of nuclear decay:

- **Alpha decay.** This involves the loss of a particle equivalent to a helium nucleus. Alpha (α) particles, being large and positively charged, do not penetrate far in living tissue, but they do cause ionisation damage and this makes them generally unsuitable for tracer studies.
- **Beta decay.** This involves the loss or gain of an electron or its positive counterpart, the positron. There are three subtypes:
 - (a) Negatron (β^-) emission: loss of an electron from the nucleus when a neutron transforms into a proton. This is the most important form of decay for radioactive tracers used in biology. Negatron-emitting isotopes of biological importance include ^3H , ^{14}C , ^{32}P and ^{35}S .
 - (b) Positron (β^+) emission: loss of a positron when a proton transforms into a neutron. This only occurs when sufficient energy is available from the transition and may involve the production of gamma rays when the positron is later annihilated by collision with an electron.
 - (c) Electron capture (EC): when a proton ‘captures’ an electron and transforms into a neutron. This may involve the production of X-rays as electrons ‘shuffle’ about in the atom (as with ^{125}I) and it frequently involves electron emission.

Table 54.1 Types of radioactivity and their properties

Radiation	Range of maximum energies (MeV*)	Penetration range in air (m)	Suitable shielding material
Alpha (α)	4–8	0.025–0.080	Unnecessary
Beta (β)	0.01–3	0.150–16	Plastic (e.g. Perspex)
Gamma (γ)	0.03–3	1.3–13 [†]	Lead

* Note that 1 MeV = $1.6 \times 10^{-13}\text{J}$.

[†] Distance at which radiation intensity is reduced to half.

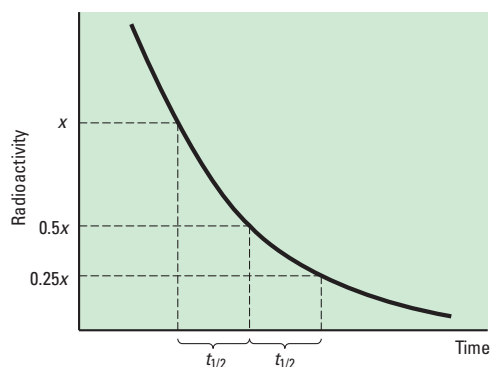


Fig. 54.1 Decay of a radioactive isotope with time. The time taken for the radioactivity to decline from x to $0.5x$ is the same as the time taken for the radioactivity to decline from $0.5x$ to $0.25x$, and so on. This time is the half-life ($t_{1/2}$) of the isotope.

- **Gamma emission.** Internal transition involves the emission of electromagnetic radiation in the form of gamma (γ) rays from a nucleus in a metastable state and always follows initial alpha or beta decay. Emission of gamma radiation leads to no further change in atomic number or mass.

Note from the above that more than one type of radiation may be emitted when a radioisotope decays. The main radioisotopes used in biology and their properties are listed in Table 54.2.

Each radioactive particle or ray carries energy, usually measured in electron volts (eV). The particles or rays emitted by a particular radioisotope exhibit a range of energies, termed an energy spectrum, characterised by the maximum energy of the radiation produced, E_{\max} (Table 54.2). The energy spectrum of a particular radioisotope is relevant to the following:

- **safety:** isotopes with the highest maximum energies will have the greatest penetrating power, requiring appropriate shielding (Table 54.1)
- **detection:** different instruments vary in their ability to detect isotopes with different energies
- **discrimination:** some instruments can distinguish between isotopes, based on the energy spectrum of the radiation produced (p. 406).

The decay of an individual atom (a ‘disintegration’) occurs at random, but that of a population of atoms occurs in a predictable manner. The radioactivity decays exponentially, having a characteristic half-life ($t_{1/2}$). This is the time taken for the radioactivity to fall from a given value to half that value (Fig. 54.1). The $t_{1/2}$ values of different radioisotopes range from fractions of a second to more than 10^{19} years (see also Table 54.2). If the

Table 54.2 Properties of some isotopes used commonly in biology. Physical data obtained from Rumble (2020)

Isotope	Emission(s)	Maximum energy (MeV)	Half-life	Main uses	Advantages	Disadvantages
^3H	β^-	0.01861	12.3 years	Suitable for labelling organic molecules in a wide range of positions at high specific activity	Relatively safe	Low efficiency of detection, high isotope effect, high rate of exchange with environment
^{14}C	β^-	0.15648	5715 years	Suitable for labelling organic molecules in a wide range of positions	Relatively safe, low rate of exchange with environment	Low specific activity
^{22}Na	β^+ (90%) + γ , EC	2.842 (β^+)	2.60 years	Transport studies	High specific activity	Short half-life, hazardous
^{32}P	β^-	1.710	14.3 days	Labelling proteins and nucleotides (e.g. DNA)	High specific activity, ease of detection	Short half-life, hazardous
^{35}S	β^-	0.167	87.2 days	Labelling proteins and nucleotides	Low isotope effect	Low specific activity
^{36}Cl	β^- , β^+ , EC	0.709 (β^-) 1.142 (β^+ , EC)	300 000 years	Transport studies	Low isotope effect	Low specific activity, hazardous
^{125}I	EC + γ	0.178 (EC)	59.9 days	Labelling proteins and nucleotides	High specific activity	Hazardous
^{131}I	β^- + γ	0.971 (β^-)	8.04 days	Labelling proteins and nucleotides	High specific activity	Hazardous

Example For ^{35}S , with a half-life of 87.2 days (Table 54.2), the fraction remaining after 28 days would be worked out as follows:
 $x = (-0.693 \times 28) \div 87.2 = -0.222522936$,
 then using eqn [54.1],

$$f = e^{-0.222522936} = 0.800496646$$

(approximately 80.0% of original activity).

Table 54.3 Relationships between units of radioactivity. For abbreviations, see text

1 Bq = 1 d.p.s.	1 Sv = 100 rem
1 Bq = 60 d.p.m.	1 Gy = 100 rad
1 Bq = 27 pCi	1 Gy \approx 100 roentgen
1 d.p.s. = 1 Bq	1 rem = 0.01 Sv
1 d.p.m. = 0.0167 Bq	1 rad = 0.01 Gy
1 Ci = 37 GBq	1 roentgen \approx 0.01 Gy
1 mCi = 37 MBq	
1 μ Ci = 37 kBq	

Example If 0.4 mL of a ^{32}P -labelled DNA solution at a concentration of $50 \mu\text{mol L}^{-1}$ (amount = $0.4 \times 50 \div 1000 = 0.020 \mu\text{mol}$) gave a count of 2490 d.p.m. (= 41.5 Bq), using eqn [54.2] this would correspond to a specific activity of $2490 \div 0.02 = 124500 \text{ d.p.m. } \mu\text{mol}^{-1}$ (or $2075 \text{ Bq } \mu\text{mol}^{-1}$).

$t_{1/2}$ is very short, as with ^{15}O ($t_{1/2} \approx 2 \text{ min}$), then it is generally impractical to use the isotope in experiments because you would need to account for the decay during the experiment and counting period.

You can calculate the fraction (f) of the original radioactivity left after a particular time (t), using the following relationship:

$$f = e^x, \text{ where } x = -0.693t/t_{1/2} \quad [54.1]$$

Note that the same units must be used for t and $t_{1/2}$ in the above equation.

Measuring radioactivity

The SI unit of radioactivity is the becquerel (Bq), equivalent to one disintegration per second (d.p.s.), but disintegrations per minute (d.p.m.) are also used ($= \text{Bq} \times 60$). The curie (Ci) is a non-SI unit equivalent to the number of disintegrations produced by 1 g of radium (37 GBq). Table 54.3 shows the relationships between these units. In practice, most instruments are not able to detect all of the disintegrations from a particular sample, i.e. their efficiency is less than 100% and the rate of decay may be presented as counts min^{-1} (c.p.m.) or counts s^{-1} (c.p.s.). Most modern instruments correct for background radiation and inefficiencies in counting, converting count data to d.p.m. Alternatively, the results may be presented as the measured count rate, although this is only valid where the efficiency of counting does not vary greatly among samples.

KEY POINT The specific activity is a measure of the quantity of radioactivity present in a known amount of the substance.

$$\text{specific activity} = \frac{\text{radioactivity (Bq, Ci, d.p.m., etc.)}}{\text{amount of substance (mol, g, etc.)}} \quad [54.2]$$

Specific activity is an important concept in practical work involving radioisotopes, since it allows interconversion of disintegrations (activity) and amount of substance (see Box 54.1).

Two SI units refer to doses of radioactivity and these are used when calculating exposure levels for a particular source. The sievert (Sv) is the amount of radioactivity giving a dose in man equivalent to 1 gray (Gy) of X-rays: $1 \text{ Gy} = \text{an energy absorption of } 1 \text{ J kg}^{-1}$. The dose received in most biological experiments is a negligible fraction of the maximum permitted exposure limit. Conversion factors from older units are given in Table 54.3.

The most important methods of measuring radioactivity for biological purposes are described below.

The Geiger–Müller (G–M) tube

This operates by detecting radiation when it ionises gas between a pair of electrodes across which a voltage has been applied (Fig. 54.2). You should use a hand-held Geiger–Müller tube for routine checking for contamination (although it will not pick up ^3H activity).

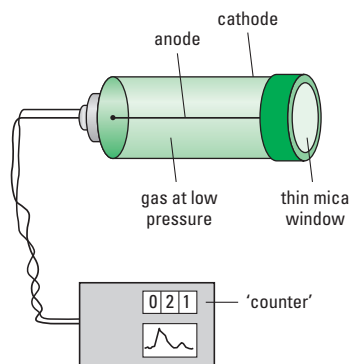


Fig. 54.2 Components of a Geiger–Müller (G–M) tube.

Box 54.1 How to determine the specific activity of an experimental solution

Suppose you need to make up a certain volume of an experimental solution, to contain a particular amount of radioactivity. For example, 50 mL of a mannitol solution at a concentration of 25 mmol L^{-1} , to contain $5 \text{ Bq } \mu\text{L}^{-1}$ – using a manufacturer's stock solution of ^{14}C -labelled mannitol (specific activity = 0.1 Ci/mmol^{-1}).

- 1. Calculate the total amount of radioactivity in the experimental solution**, in this example 5×1000 (to convert μL to $\text{mL} \times 50$ (50 mL required) = $2.5 \times 10^5 \text{ Bq}$ (i.e. 250 kBq).
- 2. Establish the volume of stock radioisotope solution required:** for example, a manufacturer's stock solution of ^{14}C -labelled mannitol contains 50 μCi of radioisotope in 1 mL of 90% v/v ethanol: water. Using Table 54.3, this is equivalent to an activity of $50 \times 37 = 1850 \text{ kBq}$. So, the volume of solution required is $250/1850$ of the stock volume, i.e. 0.1351 mL (135 μL).
- 3. Calculate the amount of non-radioactive substance required** as for any calculation involving concentration (see pp. 153, 156), e.g. 50 mL (0.05 L) of a 25 mmol L^{-1} (0.025 mol L^{-1}) mannitol (relative molecular mass 182.17) will contain $0.05 \times 0.025 \times 182.7 = 0.2277 \text{ g}$.
- 4. Check the amount of radioactive isotope to be added.** In most cases, this represents a negligible amount of substance, e.g. in this instance, 250 kBq of stock solution at a specific activity of $14.8 \times 10^6 \text{ kBq/mmol}^{-1}$ (converted from 0.4 Ci mmol^{-1} using Table 54.3) is equal to $250/14\,800\,000 = 16.89 \text{ nmol}$, equivalent to approximately 3 μg mannitol. This can be ignored in calculating the mannitol concentration of the experimental solution.
- 5. Make up the experimental solution** by adding the appropriate amount of non-radioactive substance and the correct volume of stock solution.

6. Measure the radioactivity in a known volume of the experimental solution. If you are using an instrument with automatic correction to Bq, your sample should contain the predicted amount of radioactivity, e.g. an accurately dispensed volume of 100 μL of the mannitol solution should give a corrected count of $100 \times 5 = 500 \text{ Bq}$ (or $500 \times 60 = 30000 \text{ d.p.m.}$).

7. Note the specific activity of the experimental solution: in this case, 100 μL ($1 \times 10^{-4} \text{ L}$) of the mannitol solution at a concentration of 0.025 mol L^{-1} will contain $25 \times 10^{-7} \text{ mol}$ (2.5 μmol) mannitol. Dividing the radioactivity in this volume (30000 d.p.m.) by the amount of substance (eqn [54.2]) gives a specific activity of $30000/2.5 = 12000 \text{ d.p.m. } \mu\text{mol}^{-1}$, or $12 \text{ d.p.m. nmol}^{-1}$. This value can be used:

- To assess the accuracy of your protocol for preparing the experimental solution: if the measured activity is substantially different from the predicted value, you may have made an error in making up the solution.
- To determine the counting efficiency of an instrument: by comparing the measured count rate with the value predicted by your calculations.
- To interconvert activity and amount of substance: the most important practical application of specific activity is the conversion of experimental data from counts (activity) into amounts of substance. This is only possible where the substance has not been metabolised or otherwise converted into another form; e.g. a tissue sample incubated in the experimental solution described above with a measured activity of 245 d.p.m. can be converted to nmol mannitol by dividing by the specific activity, expressed in the correct form. Thus $245/12 = 20.417 \text{ nmol mannitol}$.

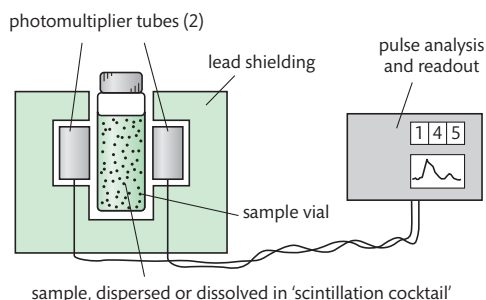


Fig. 54.3 Components of a scintillation counter. Note that in most modern instruments, all components are enclosed within a single cabinet.

The scintillation counter

This operates by detecting the scintillations (fluorescence 'flashes') produced when radiation interacts with certain chemicals called fluors (Fig. 54.3). In solid (or external) scintillation counters (often referred to as 'gamma counters') the radioactivity causes scintillations in a crystal of fluorescent material held close to the sample. This method is only suitable for radioisotopes producing penetrating radiation.

Liquid scintillation counters are mainly used for detecting beta decay and they are especially useful in biology. The sample is dispersed or dissolved in a suitable solvent containing the fluor(s) – the 'scintillation cocktail'. The radiation first interacts with the solvent, and the energy from this interaction

Correcting for quenching – find out how your instrument corrects for quenching and check the quench indication parameter (QIP) on the printout, which measures the extent of quenching of each sample. Large differences in the QIP would indicate that quenching is variable among samples and might give you cause for concern.

Liquid scintillation counting of high-energy β -emitters – β -particles with energies greater than 1 MeV (e.g. ^{32}P) can be counted in water (Čerenkov radiation), with no requirement for additional fluors.

is passed to the fluors, which produce detectable light. The scintillations are measured by photomultiplier tubes (Fig. 54.3) that turn the light pulses into electronic pulses, the magnitude of which is directly related to the energy of the original radioactive event. The spectrum of electronic pulses is thus related to the energy spectrum of the radioisotope.

Modern liquid scintillation counters use a series of electronic ‘windows’ to split the pulse spectrum into two or three components. This may allow more than one isotope to be detected in a single sample, provided their energy spectra are sufficiently different (Fig. 54.4). A complication of this approach is that the energy spectrum can be altered by pigments and chemicals in the sample, which absorb scintillations or interfere with the transfer of energy to the fluor; this is known as quenching (Fig. 54.4). Most instruments have computer-operated quench-correction facilities (based on measurements of standards of known activity and energy spectrum) that correct for such changes in counting efficiency.

Many liquid scintillation counters treat the first sample as a ‘background’, subtracting whatever value is obtained from the subsequent measurements as part of the procedure for converting to d.p.m. If not, you will need to subtract the background count from all other samples. Make sure that you use an appropriate background sample, identical in all respects to your radioactive sample but with no added radioisotope, in the correct position within the machine. Check that the background reading is reasonable (15–30 c.p.m.). Tips for preparing samples for liquid scintillation counting are given in Box 54.2.

Gamma-ray (γ -ray) spectrometry

This is a method by which a mixture of γ -ray-emitting radionuclides can be resolved quantitatively by pulse-height analysis. It is based on the fact that pulse heights (voltages) produced by a photomultiplier tube are proportional to the amounts of γ -ray energy arriving at the scintillant or

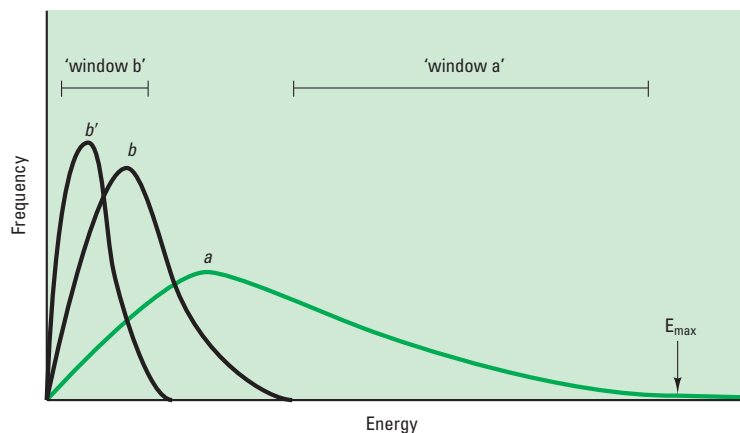


Fig. 54.4 Energy spectra for three radioactive samples, detected using a scintillation counter. Sample *a* is a high-energy β -emitter while *b* contains a low-energy β -emitter, giving a lower spectral range. Sample *b'* contains the same amount of low-energy β -emitter, but with quenching, shifting the spectral distribution to a lower energy band. The counter can be set up to record disintegrations within a selected range (a ‘window’). Here, ‘window *a*’ could be used to count isotope *a* while ‘window *b*’ could give a value of isotope *b*, by applying a correction for the counts due to isotope *a*, based on the results from ‘window *a*’. Dual counting allows experiments to be carried out using two isotopes (double labelling).

Box 54.2 How to prepare samples for liquid scintillation counting

Modern scintillation counters are very simple to operate; problems are more likely to be due to inadequate sample preparation than to incorrect operation of the machine. Common pitfalls are the following:

- **Incomplete dispersal of the radioactive compound in the scintillation cocktail.** This may lead to underestimation of the true amount of radioactivity present:
 - (a) Water-based samples may not mix with the scintillation cocktail – change to an emulsifier-based cocktail. Take care to observe the recommended limits, upper and lower, for amounts of water to be added or the cocktail may not emulsify properly.
 - (b) Solid specimens may absorb disintegrations or scintillations: extract radiochemicals using an intermediate solvent like ethanol (ideally within the scintillation vial) and then add the cocktail. Tissue-solubilising compounds such as Soluene are effective, particularly for animal material, but extremely toxic, so the manufacturer's instructions must be followed closely. Radioactive compounds on slices of agarose or polyacrylamide gels may be extracted using a product such as Protosol. Agarose gels can be dissolved in a small volume of boiling water.
 - (c) Particulate samples may sediment to the bottom of the scintillation vial – suspend them by forming a gel. This can be done with certain emulsifier-based cocktails by adding a specific amount of water.
- **Chemiluminescence.** This is where a chemical reacts with the fluors in the scintillation cocktail causing spurious scintillations, a particular risk with solutions containing strong bases or oxidising agents. Symptoms include very high initial counts that decrease through time. Possible remedies are:
 - (a) Leave the vials at room temperature for a time before counting. Check with a suitable blank that counts have dropped to an acceptable level.
 - (b) Neutralise basic samples with acid (e.g. acetic acid or HCl).
 - (c) Use a scintillation cocktail that resists chemiluminescence such as Hiconicfluor.
 - (d) Raise the energy of the lower counts detected to about 8 keV – most chemiluminescence pulses are weak (0–7 keV). This approach is not suitable for weak emitters, e.g. ^3H .

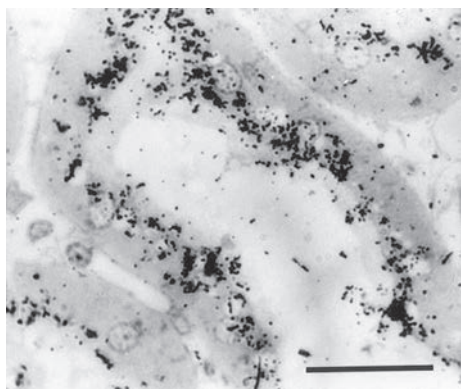


Fig. 54.5 Autoradiograph showing the localisation of a growth factor in epithelial cells of rat kidney tubules (scale bar is 10 μm). The black-silver grains are due to radioactivity from ^{125}I -labelled growth factor. From Alexander *et al.* (1998), with permission.

a lithium-drifted germanium detector. The lithium-drifted germanium detector, which is abbreviated to Ge(Li) – pronounced ‘jelly’ – provides high resolution (narrow peaks), essential in the analysis of complex mixtures.

Autoradiography

This is a method where photographic film is exposed to the isotope. It is used mainly to locate radioactive tracers in thin sections of an organism or on chromatography papers and gels, but quantitative work is possible. The radiation interacts with the film in a similar way to light, silver grains being formed in the developed film where the particles or rays have passed through. The radiation must have enough energy to penetrate into the film, but if it has too much energy the grain formation may be too distant from the point where the isotope was located to identify precisely the point of origin (for example, high-energy β -emitters). Autoradiography (Fig. 54.5) is a relatively specialised method and individual lab protocols should be followed for particular isotopes/applications.

Using radioactive isotopes in the biosciences

The main advantages of employing radioactive isotopes in biological experiments are:

- **Radioactivity is readily detected.** Methods of detection are sufficiently sensitive to measure extremely small amounts of radioactive substances.

Studying the metabolism of radiolabelled compounds – you may need to separate individual metabolites before counting, e.g. using chromatography (Chapter 48), or electrophoresis (Chapter 50).

- **Studies can be carried out on intact, living organisms.** If care is taken, minimal disruption of normal conditions will occur when radiolabelled compounds are introduced.
- **Protocols are simple** compared with equivalent methods for chemical analysis.

The main disadvantages are:

- **The ‘isotope effect’.** Molecules containing different isotopes of the same atom may react at slightly different rates and behave in slightly different ways from the natural isotope. The isotope effect is more extreme the smaller the atom and is most important for ^3H -labelled compounds of low molecular mass.
- **The possibility of mistaken identity.** The presence of radioactivity does not tell you anything about the compound in which the radioactivity is present: it could be different from the one in which it was applied, due to metabolism or spontaneous breakdown of a ^{14}C -containing organic compound.

The main types of experiments are:

- **Investigations of metabolic pathways:** a radioactively labelled substrate is added (often to an *in vitro* experimental ‘system’ rather than a whole organism) and samples taken at different time intervals. By identifying the labelled compounds and plotting their appearance through time, an indication of the pathway of metabolism can be obtained.
- **Translocation studies:** radioisotopes are used to follow the fate of molecules within an organism. Uptake and translocation rates can be determined with relative ease.
- **Ecological studies:** radioisotopic tracers provide a convenient method for determining food web interrelationships and for investigating behaviour patterns, while environmental monitoring may involve following the ‘spectral signature’ of isotopes deliberately or accidentally released.
- **Radio-dating:** the age of plant or mineral samples can be determined by measuring the amount of a radioisotope in the sample. The age of the specimen can be found using the $t_{1/2}$ by assuming how much was originally incorporated.
- **Mutagenesis and sterilisation:** radioactive sources can be used to induce mutations, particularly in micro-organisms. Gamma emitters of high energy will kill microbes and are used to sterilise equipment such as disposable Petri dishes.
- **Bioassays:** radioisotopes are used in several quantitative detection methods of value to biologists. Radioimmunoassay is described on pp. 395–6. Isotope dilution analysis works on the assumption that introduced radiolabelled molecules will equilibrate with unlabelled molecules present in the specimen. The amount of substance initially present can be worked out from the change in specific activity of the radioisotope when it is diluted by the ‘cold’ material. A method is required to purify the substance from the sample and sufficient substance must be present for its mass to be measured accurately.

Lowenthal and Airey (2005) give further details and applications.

Example Carbon dating – living organisms have essentially the same ratio of ^{14}C to ^{12}C as the atmosphere; however, when an organism dies, its $^{14}\text{C}/^{12}\text{C}$ falls because the radioactive ^{14}C isotope decays. Since we know the half-life of ^{14}C (5715 years), a sample’s $^{14}\text{C}/^{12}\text{C}$ ratio will allow us to estimate its age; e.g. if the ratio were exactly 1/8 of that in the atmosphere, the sample is three half-lives old and was formed 17 145 years before present. Such estimates carry an error of the order of 10% and are unreliable for samples older than 50 000 years, for which longer-lived isotopes can be used.

Registering for radioisotope work – in the UK, institutions must be registered for work with specific radioisotopes under the *Radioactive Substances Act* (1993).

Working under supervision with radioisotopes – in the UK, the *Ionising Radiations Act* (1985) provides details of local arrangements for the supervision of radioisotope work.

SAFETY NOTE Each new experiment should be planned carefully and experimental protocols laid down in advance so you work as safely as possible and do not waste expensive radioactively labelled compounds.

Carrying out a 'dry run' – consider doing this before working with radioactive compounds, perhaps using a dye to show the movement or dilution of introduced liquids, as this will lessen the risks of accident and improve your technique.

SAFETY NOTE The correct way to use Benchkote and similar products is with the waxed surface *down* (to protect the bench or tray surface) and the absorbent surface *up* (to absorb any spillage). Write the date in the corner when you put down a new piece. Monitor using a G-M tube and replace regularly under normal circumstances. If you are aware of spillage, replace immediately and dispose of correctly.

Working with radioactive isotopes

By law, undergraduate work with radioactive isotopes must be very closely supervised. In practical classes, the protocols will be clearly outlined, but in project work you may have the opportunity to plan and carry out your own experiments, albeit under supervision. Some of the factors that you should take into account, based on the assumption that your department and laboratory are registered for radioisotope use, are discussed below:

- **Must you use radioactivity?** If not, it may be a legal requirement that you use an alternative method.
- **Have you registered for radioactive work?** Usually, all users must register with a local Radiation Protection Supervisor. Details of the project may have to be approved by the appropriate administrator(s). You may have to have a short medical examination before you can start work.
- **What labelled compound will you use?** Radioactive isotopes must be ordered well in advance through your department's Radiation Protection Supervisor. Aspects that need to be considered include:
 - (a) The radionuclide. With many organic compounds this will be confined to ^3H and ^{14}C (but see Table 54.2). The risk of a significant 'isotope effect' may influence this decision (see p. 408).
 - (b) The labelling position. This may be a crucial part of a metabolic study. Specifically labelled compounds are normally more expensive than those that are uniformly ('generally') labelled.
 - (c) The specific activity. The upper limit for this is defined by the isotope's half-life but, below this, the higher the specific activity, the more expensive the compound.
- **Are suitable facilities available?** You will need a dedicated work area, preferably out of the way of general lab traffic and within a fume cupboard for those cases where volatile radioactive substances are used or may be produced.

In conjunction with your supervisor, decide whether your method of application will introduce enough radioactivity into the system, how you will account for any loss of radioactivity during recovery of the isotope and whether there will be enough activity to count at the end. You should be able to predict approximately the amount of radioactivity in your samples, based on the specific activity of the isotope used, the expected rate of uptake/exchange and the amount of sample to be counted. Use the isotope's specific activity to estimate whether the non-radioactive ('cold') compound introduced with the radiolabelled ('hot') compound may lead to excessive concentrations being administered. Advice for handling data is given in Box 54.1.

Safety and procedural aspects

Make sure the bench surface is one that can be easily decontaminated by washing (for example, Formica) and always use a disposable surfacing material such as Benchkote. It is good practice to carry out as many operations as possible within a Benchkote-lined plastic tray so that any spillages are contained. You will need a lab coat to be used exclusively for work with radioactivity, safety spectacles and a supply of thin latex or vinyl disposable gloves. Suitable vessels for liquid waste disposal will be required and special plastic bags for solids – make sure you know beforehand the



Fig. 54.6 Tape showing the international symbol for radioactivity.

disposal procedures for liquid and solid wastes. Wash your hands after handling a vessel containing a radioactive solution and again before removing your gloves. Gloves should be placed in the appropriate disposal bag as soon as your experimental procedures are complete.

It is important to comply with the following guidelines:

- **read and obey the local rules** for safe usage of radiochemicals
- **maximise the distance between you and the source** as much as possible
- **minimise the duration of exposure**
- **wear protective clothing** (properly fastened lab coat, safety glasses, gloves) at all times
- **use appropriate shielding** at all times (Table 54.1)
- **monitor your working area for contamination** frequently
- **mark all glassware, trays, bench work areas, etc.,** with tape incorporating the international symbol for radioactivity (Fig. 54.6)
- **keep adequate records** of what you have done with a radioisotope – the amount remaining and that disposed of in waste form must agree
- **store radiolabelled compounds appropriately** and return them to storage immediately after use
- **dispose of waste promptly** and with due regard for local rules
- **make the necessary reports about waste disposal, etc.,** to your departmental Radiation Protection Supervisor
- **clear up** after you have finished each experiment
- **wash thoroughly** after using radioactivity
- **monitor the work area and your body** when finished.

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STUDY EXERCISES

54.1 Carry out a half-life calculation. A rat dropping found in a pyramid in Egypt had a $^{14}\text{C}:^{12}\text{C}$ ratio that was 57.25% of a modern-day standard. Use this value to estimate the approximate date when the rat visited the pyramid, to the nearest century.

54.2 Practise radioactivity interconversions. Express the following values in the alternative units indicated, with appropriate prefixes as necessary. Answer to three significant figures.

- (a) 72 000 d.p.m. as Bq
- (b) 20 μCi as d.p.m.
- (c) 44 400 Bq as μCi
- (d) 63×105 d.p.m. mol^{-1} as Bq g^{-1} , for a compound with a relative molecular mass of 350
- (e) 3108 d.p.m. as pmol, for a sample of a standard where the specific activity is stated as 50 Ci mol^{-1} .

54.3 Use the concept of specific activity in calculations.

A researcher wishes to estimate the rate of uptake of the sugar galactose by carrot cells in a suspension culture. She prepares 250 mL of the cell culture medium containing 10^7 cells per mL and unlabelled galactose at a concentration of 5 mmol L^{-1} . She then 'spikes' this with $5 \mu\text{L}$ (regard this as an insignificant volume) of radioactive standard containing 55 MBq of ^{14}C -labelled galactose (regard as an insignificant concentration).

- (a) Calculate the specific activity of the galactose in the culture solution in Bq mol^{-1} .
- (b) If the total cell sample takes up $79.2 \times 10^5 \text{ Bq}$ in a 2-h period, calculate the galactose uptake rate in $\text{mol s}^{-1} \text{ cell}^{-1}$.

Answer to two significant figures.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

55 Analysing stable isotopes

Example Sodium has only one stable isotope, ^{23}Na (100.00%), giving an A_r for Na of 23.00, whereas chlorine has two stable isotopes, ^{35}Cl (75.77%) and ^{37}Cl (24.23%), giving an A_r of 35.44. Therefore, when combined as NaCl, the M_r is $23.00 + 35.44 = 58.44$.

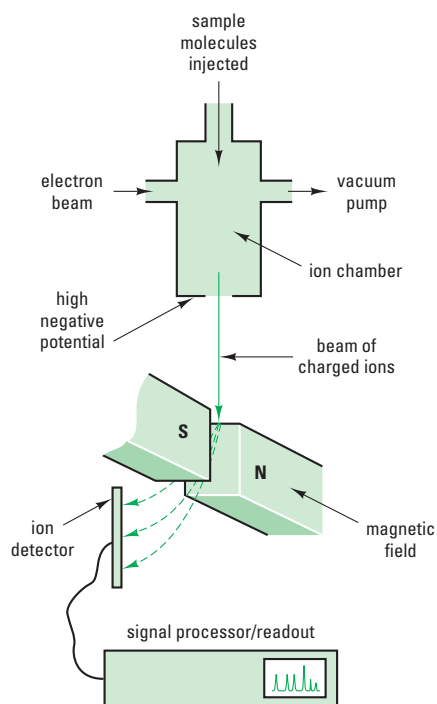


Fig. 55.1 Components of an electron impact mass spectrometer.

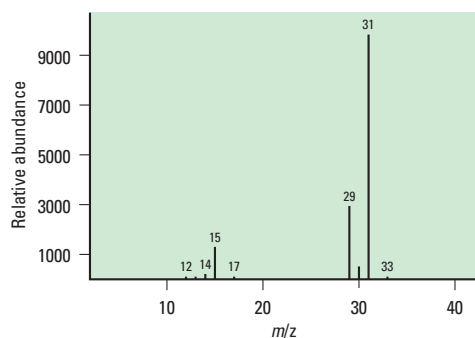


Fig. 55.2 Mass spectrum for methanol. m/z = charge ratio.

Stable isotopes of an element all have the same number of protons, but different numbers of neutrons. In contrast to radioisotopes (Chapter 54), they do not spontaneously decay to produce radiation. Stable isotopes have virtually identical gross chemical properties. When two or more stable isotopes of the same element exist in nature, they each contribute to the relative atomic mass (A_r) in direct proportion to their relative abundance.

Given their broad chemical equivalence, stable isotopes have a narrow range of practical applications in biology, and the techniques used to measure them are highly specialised and sensitive; they include mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectrometry.

Measuring stable isotopes by mass spectrometry

Typically, stable isotopes are separated and quantified in terms of their mass-to-charge ratio using some form of mass spectrometry. This technique involves the vaporisation/ionisation of a sample in a gas phase. The ions are then accelerated to specific velocities using an electric field and then separated on the basis of their different masses and charges (Fig. 55.1). Each ion of a particular mass-to-charge ratio is detected sequentially with time. The most widely used method in MS is electron impact (EI) ionisation, where the electron source is a heated metal, such as a tungsten filament, subjected to an appropriate potential gradient. The stream of electrons emitted can then interact with elements and molecules in the sample, by either:

- **electron removal** – where an electron in a bond within the sample molecule is ‘knocked out’ by bombarding electrons, producing a positively charged ion (cation); or
- **electron capture** – where addition of an electron results in the production of a negatively charged ion (anion).

The ions are then subjected to a magnetic field (Fig. 55.1) which results in their detection in proportion to their abundance in the sample. Signal processing and analysis then give a mass spectrum, such as that shown in Fig. 55.2, which can be used to characterise and identify the compound.

For metabolic studies, the technique of isotope ratio mass spectrometry (IRMS) is very useful, since it removes the need to use radioactive isotopes. The technique exploits the ability of MS to distinguish between isotopes such as ^{13}C and ^{12}C . For example, a compound containing ^{13}C will have a greater overall mass than the same compound containing ^{12}C and can therefore be differentiated within the mass spectrum produced as a result of MS. By selectively labelling a key metabolite with a stable isotope that is naturally present at low concentration (for example, ^{13}C), it is then possible to follow the fate of the metabolite by MS analysis of sequential samples. Typically, the metabolites would be combusted in an oxygen atmosphere, to give gaseous $^{13}\text{CO}_2$, followed by exposure of such gases to an EI source (Fig. 55.1); useful metabolic data can be obtained from the isotope ratio – in this instance $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$.

Using stable carbon isotopes in photosynthetic studies

Measurements of stable carbon isotopes in organic material and in the air passing over photosynthetically active plant material can be used as a probe of photosynthetic physiology. ‘Light’ and ‘heavy’ isotopes of

carbon (^{12}C and ^{13}C , respectively) have different diffusion rates within the leaf and the key photosynthetic enzyme Rubisco (ribulose biphosphate carboxylase oxygenase) also discriminates against ^{13}C , leading to changes in the isotope composition of photo-assimilated carbon compared to that of air. By measuring this discrimination, the physiological processes associated with carbon fixation and metabolism can be investigated in a time-integrated manner. Analysis of stable isotope composition makes use of mass spectrometric measurements of the ratio of heavy-to-light isotopes in the sample under investigation and in a predefined standard.

The mass spectrometer compares the mass-to-charge ratio (m/z) of the 44 and 45 masses of $^{12}\text{CO}_2$ and $^{13}\text{CO}_2$ respectively, to give the isotopic composition of (i) a sample and (ii) a standard, in terms of the ^{13}C molar abundance ratio (R), where:

$$R = \frac{[^{13}\text{CO}_2]}{[^{12}\text{CO}_2]} \quad [55.1]$$

These values are then used to calculate the isotopic composition of the sample relative to the standard as a $\delta^{13}\text{C}$ value, usually expressed in parts per thousand (‰ – note that this is a proportion, and *not* a unit – spoken as ‘per mil’). The original standard was CO_2 derived from a fossil *Belemnite* from the *Pee Dee* deposits in South Carolina (PDB standard: $R = 0.01124$). The $\delta^{13}\text{C}$ value of a sample in parts per thousand is then calculated as:

$$\delta^{13}\text{C} = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 100 \quad [55.2]$$

Isotopic composition values may also be used to calculate discrimination (Δ), which takes into account the isotopic composition of the standard, source and sample, according to the following equation:

$$\Delta = \frac{\delta_a - \delta_p}{1 + \delta_p} \quad [55.3]$$

where δ_a and δ_p are the carbon isotope discrimination values for air (source) and plant (sample) respectively, expressed in fractional terms (see worked examples in margin). In contrast to $\delta^{13}\text{C}$ values which are negative when PDB is used as the standard, values for Δ are positive for plant material, since ^{12}C is favoured over ^{13}C . The advantage in using Δ notation is that it is more straightforward to make comparisons, avoiding the possible confusion involved in discussing differences in negative $\delta^{13}\text{C}$ values. Like $\delta^{13}\text{C}$ values, Δ values are usually expressed in parts per thousand (‰). Values for Δ values in C_3 plants, where Rubisco is the main carboxylating enzyme, usually fall within the range 16–25‰, since Rubisco shows substantial discrimination against ^{13}C . In contrast, Δ values for C_4 plants, where PEP carboxylase is the main carboxylating enzyme, are lower – typically in the range 3–7‰ – since PEP carboxylase shows minimal discrimination against ^{13}C .

Research studies of hydrogen, oxygen and nitrogen isotope ratios have further extended the range of tools available to physiologists to investigate topics including plant water relations and nitrogen economy at the individual plant and ecosystem levels. Since the stable carbon isotope ratio of a plant is a broad measure of the overall balance between carbon uptake (photosynthesis) and water loss (transpiration), Δ is found to vary

Example Using eqn [55.1], for air, with $^{13}\text{CO}_2$ at $0.144 \mu\text{mol l}^{-1}$ and $^{12}\text{CO}_2$ at $12.915 \mu\text{mol l}^{-1}$, gives a molar abundance ratio, $R = 0.144 \div 12.915 = 0.01115$ (to four significant figures).

Example Using eqn [55.2], for air, where $R = 0.01115$, $\delta^{13}\text{C} = (0.01115 - 0.01124) \div 0.01124 \times 1000 = -8.0\%$ (to one decimal place). $\delta^{13}\text{C}$ for plant tissue where Rubisco is the main carboxylating enzyme is *more negative* than $\delta^{13}\text{C}$ for C_4 plant tissue, since Rubisco discriminates strongly against ^{13}C (e.g. $R = 0.01095$, $\delta^{13}\text{C} = -25.8\%$). $\delta^{13}\text{C}$ for C_4 plant tissue where phosphoenolpyruvate (PEP) carboxylase is the main carboxylating enzyme is *less negative* than $\delta^{13}\text{C}$ for C_3 plant tissue, since this enzyme shows little discrimination against ^{13}C , and the main effect is due to diffusion within the leaf (e.g. $R = 0.01111$, $\delta^{13}\text{C} = -11.6\%$).

Example Using eqn [55.3], Δ for C_3 plant tissue with a $\delta^{13}\text{C}$ of -25.8% is $[-0.008 - (-0.0258)] \div [1 + (-0.0258)] = 0.0182714 = 18.2\%$. Using eqn [55.3], Δ for C_4 plant tissue with a $\delta^{13}\text{C}$ of -11.5% is $[-0.008 - (-0.0115)] \div [1 + (-0.0115)] = 0.0035407 = 3.5\%$.

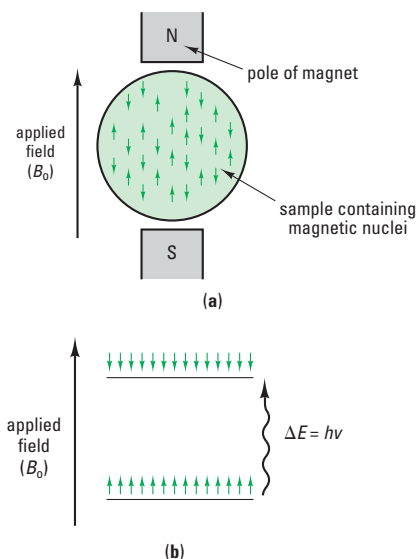


Fig. 55.3 Effect of an applied magnetic field, B_0 , on magnetic nuclei. (a) Nuclei in magnetic field have one of two orientations – either with the field or against the field (in the absence of an applied field, the nuclei would have random orientation). (b) Energy diagram for magnetic nuclei in applied magnetic field.

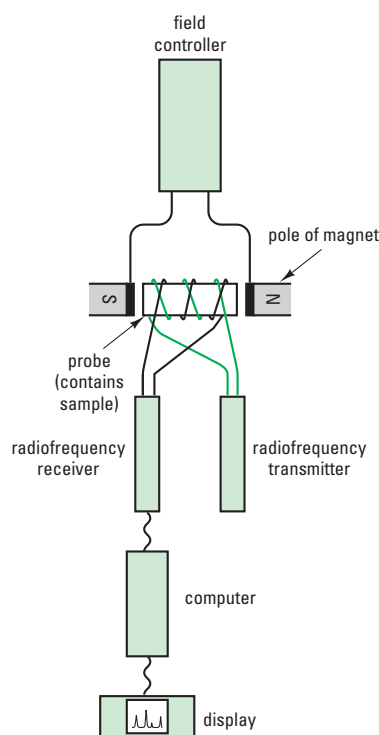


Fig. 55.4 Components of an NMR spectrometer.

with changes in transpiration rate, with a lower value corresponding to a higher water use efficiency. It has been proposed that concurrent analysis of $^{18}\text{O}/^{16}\text{O}$ ratios can enable researchers to interpret variations in Δ in terms of water-use efficiency: this can be useful in plant breeding studies, where improved water-use efficiency results in closely coupled ^{13}C and ^{18}O signals.

Analysing stable isotopes by NMR analysis

Electromagnetic radiation at radiofrequencies of 1–500 MHz can be used to identify and monitor biological compounds and metabolic processes. This is possible because of differences in the magnetic states of atomic nuclei of different stable isotopes, involving very small transitions in energy level. The atomic nuclei of isotopes of many elements are magnetic, because they are charged and have spin. Typical stable isotopes that are magnetic include ^1H , ^{13}C , ^{14}N , ^{15}N , ^{19}F and ^{31}P . When these nuclei interact with a uniform external magnetic field, they behave like tiny compass needles and align themselves in a direction either (i) parallel or (ii) antiparallel to the external magnetic field. The two orientations have different energies, with the parallel direction having a lower energy than the antiparallel direction (Fig. 55.3). The energy difference between these two levels corresponds to a precise electromagnetic frequency.

When a sample containing an isotope with a magnetic nucleus is placed in a magnetic field and exposed to an appropriate radiofrequency in an NMR spectrometer (Fig. 55.4), transitions between the energy levels of magnetic nuclei will occur when the energy gap and the applied frequency are in *resonance* (i.e. when they are matched exactly). This frequency is a characteristic of the particular isotope and can be used to study the isotope in biological systems. Typically, this is described in terms of the ‘chemical shift’ of the nuclei involved, which is a field-independent dimensionless value measured against the resonance frequency of a reference compound (for example, tetramethylsilane for ^1H and ^{13}C NMR), as:

$$\frac{\text{resonance frequency of sample} - \text{resonance frequency of reference}}{\text{resonance frequency of the reference}} \quad [55.4]$$

Because the difference in frequency (numerator in [55.4]) is very small in relation to the resonance frequency of the reference (denominator in [55.4]), chemical shifts are usually expressed in parts per million (ppm).

The resonance frequency of magnetic nuclei is affected by the presence of shielding due to small secondary fields generated by electrons in their vicinity, giving rise to different chemical shifts for the same isotope in different parts of a molecule. By convention, the chemical shift is positive if the nucleus is less shielded than the reference compound and negative if it is more shielded. The end result is an NMR spectrum where individual magnetic nuclei produce a ‘signal’ with peaks representing the different locations within a molecule.

Biomolecular applications of NMR

The sensitivity of NMR has improved dramatically with the development of more powerful magnets. Details of the major uses of various magnetic nuclei are given in Table 55.1. The principal applications include:

- **studies of the structure and function of macromolecules and biological systems**, such as membranes

Table 55.1 The relative merits and disadvantages of various magnetic nuclei in biomolecular studies.

Nucleus	Relative sensitivity	Natural abundance	Comments
^1H	100	99.98%	Multiple, but specific, spectral lines are obtained for individual biomolecules. For mixtures, the ubiquitous occurrence of ^1H gives complex, overlapping signals that are often difficult to interpret. Gives a large solvent peak with aqueous samples (can be avoided by using D_2O as solvent). Mainly used for structural studies of pure macromolecules. Essential for MRI.
^{31}P	6.6	100%	Very useful for studies on living systems, with narrow resonance peaks and a wide range of chemical shifts for different molecules. Spectra are simpler and easier to interpret than for ^1H , but are not as distinctive: different compounds may give similar ^{31}P spectra. Several important P-containing compounds (including ATP, ADP and inorganic phosphate) can be detected in intact cells – useful in bioenergetic studies.
^{13}C	0.016	1.1%	Gives narrow signals and a wide range of chemical shifts. Resolution is better than for ^1H , and a wide range of organic biomolecules can be detected. Low natural abundance gives low sensitivity, extending the time required to accumulate spectra. However, low natural abundance also means that specific metabolites can be selected for ^{13}C isotope enrichment, allowing particular metabolic pathways to be investigated, e.g. carbon assimilation.

Using NMR – in contrast to most conventional metabolic studies, NMR is non-invasive and the time course of metabolic reactions can be followed using a single experimental subject or preparation, eliminating variation between samples.

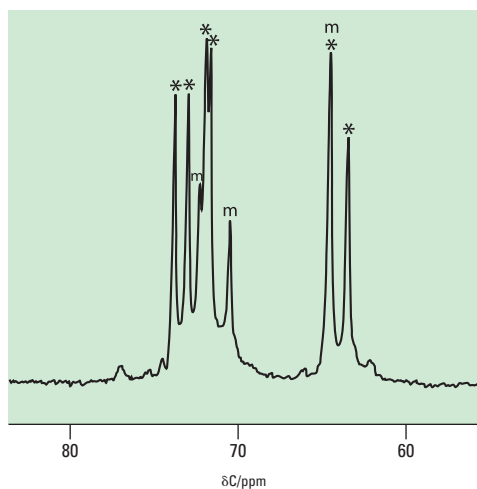


Fig. 55.5 A ^{13}C NMR spectrum of *Himanthalia elongata*, with asterisks showing the peaks corresponding to individual nuclei within the carbohydrate alditol. m = mannitol (Courtesy J.A. Chudek).

- **metabolic investigations on living organisms**, including humans, since NMR can be used to obtain a ‘fingerprint’ of a particular molecule, and changes in the intensity of spectra can be used for kinetic studies (for example, Fig. 47.3); this involves use of ‘surface coils’ as sources of radiation, or placing the organism within the core of an electromagnet
- **measurement of intracellular pH** by determination of the chemical shift of the ^{31}P phosphate peak, as this changes with pH in a predictable manner (Fig. 47.3 p. 342)
- **magnetic resonance imaging (MRI)**, which is a form of proton NMR that uses a field gradient (as opposed to a uniform field) to produce signals that are translated by computers into anatomical images (Chary and Govil, 2008). Body tissues have different water contents (typically 60–90% w/w). Signals arising from protons in water in different tissues can be used to differentiate between tissues (for example, ‘grey’ and ‘white’ matter in the brain), and between normal tissue and tumours. Fat deposits can be detected, owing to the difference between the ^1H signal from fatty acids and that of the water in the surrounding tissue.

^{13}C NMR analysis is particularly useful in photosynthetic studies; for example, in identifying the most abundant organic low molecular weight solutes in plants, and for studying how the concentrations of these solutes are adjusted in response to salt and water stress. The relative insensitivity of this approach, with ^{13}C representing only 1.1% of the naturally occurring carbon atoms in biomolecules, becomes an advantage in such studies. Only those soluble organic molecules that are present in large quantities within a cell will be detected, giving a spectrum that typically shows signals from only one or two solutes. As an example, Fig. 55.5 shows a natural abundance ^{13}C NMR spectrum of the seaweed *Himanthalia elongata*, which was the first report of the carbohydrate alditol in any living organism (Chudek *et al.*, 1984) – previous chromatographic studies had failed to separate this compound from other isomers, such as mannitol. The relatively low natural abundance of ^{13}C also means that ‘labelling’ studies can be carried out, for example, using $^{13}\text{CO}_2$, to follow the metabolism of photosynthetically fixed C within the plant.

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[Covers the basics of magnetic resonance imaging in medicine.]

STUDY EXERCISES

55.1 Test your understanding of the terminology of stable isotopes. What do the following terms/acronyms mean? (a) NMR; (b) MRI; (c) EI (d) chemical shift; (e) electron capture.

55.2 Explain the operating principles of a nuclear magnetic resonance (NMR) spectrometer. Make sure that your explanation covers the major components of an NMR spectrometer.

55.3 Calculate the molar abundance ratio ($R = [^{13}\text{CO}_2]/[^{12}\text{CO}_2]$ and $\delta^{13}\text{C}([R_{\text{sample}} - R_{\text{standard}}]/R_{\text{standard}} \times 1000)$ of a sample. Determine R and $\delta^{13}\text{C}$ for an air sample with $[^{13}\text{CO}_2] = 0.152 \mu\text{mol L}^{-1}$ and $[^{12}\text{CO}_2]$ at $12.825 \mu\text{mol L}^{-1}$, and PDB standard of $R = 0.011\,24$.

Answers to these study exercises are available at go.pearson.com/uk/he/resources



Assaying biomolecules and studying metabolism

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56 Analysing biomolecules

Definitions

Accuracy – the closeness of an individual measurement, or a mean value based on a number of measurements, to the true value.

Detection limit – the minimum concentration of an analyte that can be detected at a particular confidence level.

Drift – ‘baseline’ movement in a particular direction: drift can be a problem between analyses (e.g. using a spectrophotometer for colorimetric analysis), or for a single analysis (e.g. when separating biomolecules by chromatography).

Noise – random fluctuations in a continuously monitored signal.

Precision – the extent of mutual agreement between replicate data values for an individual sample.

Quality assurance – procedures to monitor, document and audit a process against a set of criteria, usually defined externally (e.g. ISO 9000), to confirm that the process meets the specified requirements.

Quality control – internal procedures designed to ensure that the performance of a process meets a particular standard, e.g. analysis of a test sample containing a known amount of a substance, to confirm that the measurement falls within an acceptable range of the true value.

Range of measurement – the range of values from the detection limit to the upper concentration at which the technique becomes inaccurate or imprecise.

Replicate – repeated measurement.

Selectivity – the extent to which a method is free from interference owing to other substances in the sample.

Sensitivity – the ability to discriminate between small differences in analyte concentration.

Validation – the process of checking the accuracy and precision of a particular analytical method in relation to specific standards, using an appropriate reference material containing a known amount of analyte.

Detecting, quantifying and determining the roles of organic compounds is central to most areas of biomolecular sciences, including biochemistry, genetics, and physiology. Some authorities estimate the number of discrete natural compounds with M_r of less than 300 as upwards of 20×10^6 , many of which will be present only at nanomolar concentrations. As a result, separating, identifying and measuring a specific biomolecule from a biological sample can be challenging. It generally requires that you detect one or more chemical or physical properties of the test substance (analyte) following several purification steps.

There are two principal approaches:

1. **Qualitative analysis** – where a sample is assayed to determine whether a biomolecule is present or absent. As an example, a blood sample might be analysed for a particular drug or a specific antibody (p. 392), or a bacterial cell might be probed for a nucleic acid sequence (p. 517).
2. **Quantitative analysis** – where the quantity of a particular biomolecule in a sample is determined, either as an *amount* (for example, as grams, or moles) or in terms of its *concentration* in the sample (for example, as g L^{-1} , or mol m^{-3}). Thus, an oligosaccharide might be determined as g plant^{-1} , or nmol cell^{-1} , or a blood sample might be analysed to determine its pH ($-\log_{10} [\text{H}^+]$), alcohol concentration in mg mL^{-1} , or glucose concentration in mmol L^{-1} . Skoog *et al.* (2017) give details of methods.

Your choice of approach will be determined by the purpose of the investigation and by the level of accuracy and precision required (p. 189). Many of the basic quantitative methods described in Chapters 57–61 rely on chemical reactions of the analyte and involve assumptions about the nature of the test substance and the lack of interfering compounds in the sample: such assumptions are unlikely to be wholly valid at all times. If you need to make more exacting measurements of a particular analyte, it may be necessary to separate it from the other components in the sample, for example, using chromatography (Chapter 48), ultracentrifugation (Chapter 44), or electrophoresis (Chapters 50 and 51), and then identify the separated components, for example, using spectroscopic methods (Chapters 46 and 47). However, each stage in the separation and purification procedure may introduce further errors and/or loss of sample, as described in detail for protein purification in Chapter 61.

KEY POINT In general, you should aim to use the simplest procedure that satisfies the purpose of your investigation – there is little value in using a complex, time-consuming or costly analytical procedure in a study where a high degree of accuracy is not required.

Identification of any chemical substance requires specific information about its molecular structure. For some chemicals and techniques, this is straightforward, as unambiguous signals are produced, for example, absorption spectra of metal ions. In cases where the probability of interference by other compounds is minimal, a purification step may not

Evaluating a new method – results from a novel technique can be compared with an established ‘standardised’ technique by measuring the same set of samples by each method and analysing the results by correlation (pp. 611–14).

Table 56.1 Some examples of colorimetric assays

Analyte	Reagent/wavelength
Amino acids	ninhydrin/540 nm
Proteins	biuret/520 nm Folin-Ciocalteu/600 nm
Carbohydrates	anthrone/625 nm
Reducing sugars	dinitrosalicylate/540 nm
DNA	diphenylamine/600 nm
RNA	orcinol/660 nm

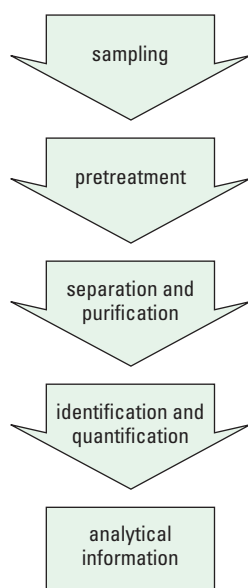


Fig. 56.1 The stages of chemical analysis. For the major classes of biomolecules, techniques for separation, purification, identification and quantification are covered in Chapters 57–61.

be necessary. There may be difficulties in identifying organic compounds owing to the possibility of isomers providing similar signals. An additional complication in biological and environmental samples is that these compounds are always present in complex mixtures, so purification is generally essential. Consequently, an element of uncertainty is present in any analytical technique.

Most of the routine methods based on chemical analysis are destructive, since the analyte is usually converted to another substance which is then assayed, for example, in colorimetric assays of the major types of biomolecules (Table 56.1). In contrast, many of the analytical methods based on physical properties are non-destructive (for example, the intrinsic absorption and emission of electromagnetic radiation in spectrophotometry, p. 338, or nuclear magnetic resonance techniques, p. 165). Non-destructive methods are often preferred, as they allow the further characterisation of a particular sample. Most biological methods are destructive: bioassays are often sensitive to interference and require validation.

Sampling for quantitative analysis

Prior to the analysis steps, you will need to obtain a sample of source material and carry out any necessary pretreatments (Fig. 56.1) as part of the process. There will be few sampling issues for qualitative analysis other than the correct identification of the organism used (see Chapters 36 and 37). However, if you are carrying out quantitative analysis, you may need to ensure your sample is representative and also to be able to define its physiological status. Aspects you may need to consider include:

- **identification of species, strain or cell line, as appropriate**
- **age, sex, condition or experimental treatment**
- **date, time and location of sampling**
- **number of samples/sub-samples taken**
- **any other features relevant to your work** (for example, a measure of pollution).

These aspects should always be specified in your Materials and methods section.

Accounting for errors in quantitative analysis

Before using a particular procedure, you should consider its possible limitations in terms of:

- **measurement errors, and their likely magnitude:** these might include processing errors (for example, in preparing solutions and making dilutions), instrumental errors (for example, a pH electrode that has not been set up correctly), calibration errors (for example, converting a digital readout to give an analyte concentration) and errors because of the presence of interfering substances
- **sampling errors:** these may occur if the material used for analysis is not representative, for example, owing to biological differences between the individual organisms used in the sampling procedure.

Replication will allow you to make quantitative estimates of several potential sources of error; for example, repeated measurements of the same sample can provide information on the precision of the analytical method,

Choosing a sampling strategy – obtaining a truly representative sample from the field is a complex matter (see Jones *et al.*, 2021). Likewise, allocation of specimens to experimental treatments should be carefully considered (Chapter 29). You should ensure that any procedure used is unbiased and that it provides material relevant to your investigation.

Interpreting results from ‘spiked’ samples – remember that such procedures tell you nothing about the *extraction efficiency* of biomolecules from a particular sample, e.g. during homogenisation (Chapter 42).

Selecting an analytical method.

Criteria include:

- the required level of accuracy and precision
- the number of samples to be analysed
- the amount of each sample available for analysis
- the physical form of the samples
- the expected concentration range of the analyte in the samples
- the sensitivity and detection limit of the technique
- the likelihood of interfering substances
- the speed of analysis
- the ease and convenience of the procedure
- the skill required by the operator
- the cost and availability of the equipment.

for example, by calculating the coefficient of variation (p. 596), while measurements of several different samples can provide information on biological and sample variability, for example, by calculating the standard deviation (Chapter 76). Analyses of different sets of samples at different times (for example, on different days) can provide information on ‘between batch’ variability, as opposed to ‘within batch’ variability (based on a single set of analytical data).

The reliability of a particular method can be assessed by measuring ‘standards’ (sometimes termed ‘controls’). These are often prepared in the laboratory by adding a known amount of analyte to a real sample (this is often termed ‘spiking’ a sample), or by preparing an artificial sample containing a known amount of analyte along with other relevant components (for example, the major sample constituents and possible interfering substances). In many instances, several standards (including a ‘blank’ or ‘zero’) are assayed to construct a ‘standard curve’, which is then used to convert sample measurements to amounts of analyte (see Chapter 43 for details). Such standard curves form the basis of many routine laboratory assays: while hand-drawn linear calibration curves are sufficient for basic assays, more complex curves are often fitted to a particular mathematical function using a computer program (for example, bioassays). Standards can also be used to check the calibration of a particular method; a mean value based on repeated measurements of an individual ‘standard’ can be compared with the true value using a modified *t*-test (eqn [76.1]), in which there is only one standard error term, i.e. that associated with the measured values.

Validation of a particular method can be important in certain circumstances, for example, in a forensic science or a clinical biochemistry laboratory, where particular results can have important implications. Such laboratories operate strict validation procedures, including: (i) adherence to standard operating procedures for each analytical method; (ii) calibration of assays using certified reference materials containing a known amount of analyte and traceable to a national reference laboratory; (iii) effective systems for internal quality control and external quality assurance (p. 419); (iv) detailed record-keeping, covering all aspects of the analysis and recording of results. Although such rigour is not required for routine analysis, the general principles of standardisation, calibration, assessment of performance and record-keeping are equally valid for all analytical work.

If you are using a completely novel technique, you can evaluate it by comparing results with an established ‘standard’ technique: you would assay the same samples by both methods and analyse the results by correlation (p. 611–14).

Text references

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STUDY EXERCISES

56.1 Check your understanding of the fundamental principles of biomolecular analysis. Distinguish between each of the following pairs of terms:

- (a) qualitative analysis and quantitative analysis;
- (b) sensitivity and selectivity;
- (c) accuracy and precision;
- (d) validation and replication;
- (e) noise and drift.

Which meter has:

- (a) the lower detection limit?
- (b) the wider linear dynamic range?
- (c) the greater accuracy?
- (d) the better level of precision?
- (e) the smaller sample volume?

56.2 Research sources of calibration standards and reference materials. Using the web, find a supplier of a validated reference standard for the following:

- (a) cortisol in serum;
- (b) cotinine in urine.

56.3 Compare equipment specifications. The table (alongside) gives operational details for two glucose meters, A and B.

Performance of two glucose meters

Aspect	Meter A	Meter B
Units of measurement	mmol L ⁻¹	mg dL ⁻¹
Detection limit	0.10	1.00
Linearity	to 20.0	to 250.0
Accuracy	±0.050	±1.50
Relative standard deviation (%)	3.5	2.5
Sample volume	40 µL	0.05 mL

Answers to these study exercises are available at go.pearson.com/uk/he/resources

57 Assaying amino acids, peptides and proteins

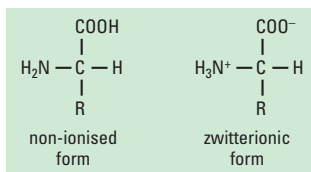


Fig. 57.1 Structure of α -amino acids. R = side chain (see Table 57.1 for examples). The α -carbon is that which connects the amino group to the acid carboxyl ($-\text{COOH}$) group. This is the D (= R) configuration; in the R (= S) isomer, the positions of the amino and carboxyl groups would be swapped.

Table 57.1 The 20 amino acids incorporated into protein, grouped according to their side chains. Those marked * are regarded as essential amino acids: they cannot be synthesised by humans and must be obtained in the diet. Other amino acids may be found when a protein is hydrolysed, owing to post-translational modification (for example, hydroxyproline).

Name	Three-letter code	Capital letter code
Aliphatic side chains		
Glycine	gly	G
Alanine	ala	A
Valine*	val	V
Leucine*	leu	L
Isoleucine*	ile	I
Aromatic side chains		
Phenylalanine*	phe	F
Tyrosine	tyr	Y
Tryptophan*	trp	W
S-containing side chains		
Cysteine	cys	C
Methionine*	met	M
Side chains with -OH groups¹		
Serine	ser	S
Threonine*	thr	T
Basic side chains		
Histidine*	his	H
Lysine*	lys	K
Arginine	arg	R
Acidic side chains		
Aspartate	asp	D
Glutamate	glu	E
Amide side chains		
Asparagine	asn	N
Glutamine	gln	Q
Cyclic structure (imino acid)		
Proline	pro	P

¹tyr also has an OH group.

All amino acids contain an amino ($-\text{NH}_2$) and carboxyl ($-\text{COOH}$) group, together with a side-chain (Fig. 57.1; Table 57.1). Roles for free amino acids in physiology include:

- **macromolecule component** – mainly in peptides and proteins; collagen, for example, consists chiefly of pro, lys and gly
- **signalling** – for example, ala, gln and ser are involved in appetite control
- **osmoregulant** – for example, gly is used as an osmoticum by certain microbes
- **detoxicant** – for example, the relatively non-toxic gln and gly are synthesised to remove excess ammonia for pH balance.

Peptides and proteins are linear polymers, formed by the linkage of α -amino acids via peptide bonds (Fig. 57.2) to create polypeptide chains. The 20 amino acids most commonly incorporated into proteins are listed in Table 57.1. Biological roles for peptides and proteins include:

- **structure** – for example, keratin, collagen
- **enzymic catalysis** – for example, amylase, trypsin
- **transportation** – for example, haemoglobin, ion pumps and channels
- **immune systems** – for example, immunoglobulins, interferons
- **hormone messenger** – for example, oxytocin, vasopressin.

KEY POINT Proteins and their constituents serve vital functions in all living organisms. To understand their role, you need to take account of their chemical configuration, how this is encoded by genes, and how they contribute to organismal structure and metabolism. Identification, quantification and structural analysis are essential parts of that process.

Understanding the structure and properties of amino acids, peptides and proteins

Amino acids

There is no agreed formal classification of amino acids but, in terms of protein structure and function, the side chains of amino acid residues can be grouped into:

- **those that carry a charge** (for example, asp, glu, lys, arg, his) – they may participate in electrostatic interactions and charge–dipole (hydrophilic) interactions
- **those that contain O, N or S** (for example, ser, met, thr, asn, gln, cys, tyr) – they can participate in hydrogen bonding and hydrophilic interactions
- **those that are hydrocarbon in nature** (for example, ala, leu, val, ile, phe, trp) – they may participate in hydrophobic interactions
- **cysteine** – this amino acid can form intra- and inter-polypeptide disulfide bridges.

Distinguishing between stereoisomers – there are two conventions:

- The R- (from Latin *recto*, 'on the right') and S- (from Latin *sinister*, 'on the left') convention involves prioritising atoms or groups bonded to an asymmetric carbon atom in order of their atomic number. Using a 3D model with the smallest atom or group pointing away from you, note the size of the remaining three groups or atoms. If the configuration in order of increasing size is clockwise, this is termed the R-configuration; if the order is anticlockwise, this is the S-configuration.
- The older 'D-' (from Latin *dextro*, or right) and 'L-' (from Latin *laevo*, or left) terminology is retained for some biomolecules, and involves the positioning of key chemical groups in relation to others when described using a Fischer projection (see p. 423)

Note that the two terminologies do not always coincide (i.e. R is not always D, and S is not always L).

Categorising polymeric forms of peptides – these are differentiated by chain length:

Oligopeptides – a very short chain, typically containing 2–20 amino acid residues. These are nutrient stores in some microbes and can act as neurotransmitters/hormones in eukaryotes; others are potent inhibitors/toxins, such as microcystin.

Peptides – polymers of up to 50 amino acid residues. These can be structural elements, but also have roles as hormones in animals, such as insulin and oxytocin.

Polypeptides and proteins – longer polymers of over 50 amino acid residues. Structural proteins and enzymes can consist of one or more polypeptides and may exist in simple or conjugated forms.

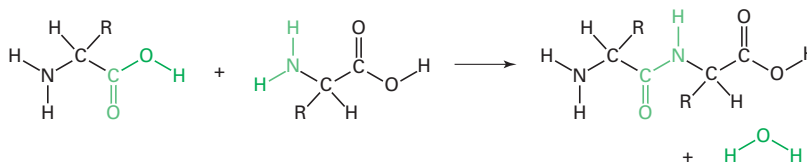


Fig. 57.2 Formation of a peptide bond between two amino acids. Water is released in this condensation reaction.

In all amino acids apart from gly, the α -carbon atom is asymmetrical, resulting in stereoisomers (Fig. 57.1). Most life scientists use the D- and L- nomenclature (Fischer convention) when referring to stereoisomers of amino acids, although the R- and S- convention (p. 424) can be a useful alternative when considering enzymatic reaction mechanisms. While most of the chemical properties of stereoisomers are identical, enzymes (including those involved in protein synthesis) can distinguish between them and, with the exception of a few bacterial metabolites, only the L-isomers of amino acids are normally involved in protein synthesis.

Polypeptides and proteins

In these polymers, the term 'primary structure' refers to the linear sequence of amino acid residues along the chain: ultimately this determines the 3D shape of the molecule (its secondary and tertiary structure) and hence its physical and biological properties (Fig. 57.3). Significantly, catalysis by enzymes (Chapter 62) is a function of this 3D arrangement.

The diversity of protein structure must be taken into account when you evaluate analytical methods. Globular proteins (for example, albumin, antibodies) are relatively soluble in dilute salt solutions, while fibrous proteins (for example, collagens, keratin) are typically insoluble, unless hydrolysed (for example, using $6 \text{ mol L}^{-1} \text{ HCl}$). Conjugated proteins (for example, metalloproteins, glycoproteins, nucleoproteins) also include non-amino acid components, which may influence your choice.

Extracting and separating amino acids, peptides and proteins

Amino acids

You can extract these compounds from homogenised cells using perchloric acid or hydrochloric acid (HCl) diluted in water or ethanol. This is usually followed by removal of cell debris and proteins by centrifugation (Chapter 44). A typical sequence for amino acid extraction from tissue might be:

1. Adding $0.1 \text{ mol L}^{-1} \text{ HCl}$ as solvent.
2. Homogenisation in a blender (p. 307).
3. Deproteinisation with acetonitrile (2.5:1 ratio) followed by centrifugation at 10 000 rpm.

Peptides and proteins

You should generally start by selecting a convenient source in which the peptide or protein of interest is known to be abundant. Typical sources can be animal tissues (for example, heart, kidney or liver) obtained from an abattoir or from laboratory animals, plant material, or microbial cells from a laboratory culture.

Definitions

Simple protein – a protein composed entirely of amino acid residues.

Conjugated protein – a protein containing a non-amino acid component (the prosthetic group), in addition to a polypeptide component (the apoprotein).

Glycoproteins – compounds consisting of proteins covalently attached to carbohydrate residues in post-translational modifications of the protein constituents.

Lipoproteins – globular, micelle-like particles consisting of a non-polar core of triacyl-glycerol and cholesterol esters surrounded by a coating of proteins, phospholipids and cholesterol; involved in the transport of lipids in blood.

Obtaining source material for protein purification – an important advance has been the use of genetically engineered organisms, designed to produce large amounts of a particular protein.

Unless the protein is extracellular, you will need to disrupt the cells in the source material and homogenise them by one of the methods described in Chapter 42. Following homogenisation, peptides and proteins from the cytosol or extracellular fluid will normally be present in a soluble form, but membrane-bound proteins and those within organelles will require further treatment. You will need to render membrane-bound proteins soluble by using organic solvents or detergents. You can use differential centrifugation (pp. 320, 440) to isolate specific organelles which will provide a degree of purification. Once the protein of interest is in a soluble form, you should remove any particulate material in the extract, for example, by centrifugation or filtration.

Note that once you have released a peptide or protein from its intracellular environment, it will be exposed to potentially adverse conditions that may result in denaturation and permanent inactivation; for example, a lower pH, an oxidising environment, or exposure to lysosomal proteases. During homogenisation, and in some or all of the purification steps, the buffers you use should contain reagents that counteract these potentially damaging effects (see Chapter 42). Fewer precautions may be needed with extracellular enzymes. In most cases, you should carry out the initial procedures at 4 °C to minimise the risk of proteolysis.

You can use both physical and chemical properties of peptides and proteins in their separation and analysis. For separation, the following properties can be exploited:

- **Net charge** – proteins and peptides differ in the types and number of amino acids with ionisable groups in their side chains. The ionisation of these side chains is pH-dependent, resulting in variation of net charge with pH (p. 364). You can exploit this property in the techniques of electrophoresis (Chapter 51), isoelectric focusing (Chapter 52) and ion-exchange chromatography (Chapter 48).
- **Water solubility and surface hydrophobicity** – you can exploit these properties respectively in ammonium sulfate precipitation, Chapter 61) and hydrophobic interaction chromatography (Chapter 48).
- **Density** – most proteins have a density of about 1.33 kg L⁻¹, but lipoproteins have a lower density. You can use this characteristic to separate lipoproteins from other classes of protein, or to subdivide the various classes of lipoproteins using centrifugation techniques (Chapter 44).

The practical procedures involved in the purification of a particular protein from a biological sample are considered in further detail in Chapter 61.

Detecting and quantifying amino acids, peptides and proteins

Amino acids

The primary amino group of amino acids will react with ninhydrin to give a purple-coloured product – you can use this reaction for qualitative assay, for example, to detect the presence of individual amino acids in chromatography or for quantitative colorimetric assay by measuring the absorbance at 570 nm using a spectrophotometer. Note that different amino acids give different amounts of coloured product on reaction with ninhydrin, so careful standardisation is needed. The secondary amino groups of the cyclic imino acids hydroxyproline and proline give yellow products with ninhydrin and are assayed at 440 nm.

Using alternative methods for quantifying proteins/peptides – these include determination of the total amount of nitrogen in solution (e.g. using the Kjeldahl technique) and calculating the protein content, assuming a nitrogen content of 16%. An alternative approach is to precipitate the protein (e.g. using trichloroacetic acid, tannic acid or salicylic acid) and then measure the turbidity of the resulting precipitate (using a nephelometer, or a spectrophotometer).

Preparing standard (calibration) curves for amino acid and protein determinations – see Chapter 43 for practical guidance.

The other features of amino acids most commonly exploited for quantitative analysis are:

- **the peptide bond**, for example, in the biuret reaction (Box 57.1)
- **the phenolic group of tyr and the indole group of trp**, which react with the oxidising agents phosphotungstic and phosphomolybdic acids in the Folin–Ciocalteu reagent to produce a blue colour; this is combined with the biuret reaction in the Lowry method (Box 57.1)
- **dye binding to hydrophobic regions**, for example, Bradford assay (Box 57.1)
- **the primary amino groups of lys residues, the guanidino group of arg residues and the N-terminal amino acid residue** will react with ninhydrin, allowing colorimetric assay similar to the qualitative method described above for isolated amino acids. A more sensitive method uses the reaction of these groups with fluorescamine to give an intensely fluorescent product, which you can measure by spectrofluorimetry (p. 448).

Peptides and proteins

Methods of quantification typically rely on similar chemical properties to those used for amino acids, including:

- **chemical reactions that are relatively specific to peptide bonds** (for example, the Biuret method)
- **UV absorbance of aromatic amino acid residues of phe, tyr and trp** (for example, the Warburg–Christian method)
- **colorimetric reactions when tyr and trp residues are oxidised** (for example, the Lowry method)
- **dye-binding** (for example, the Bradford method).

Details of the procedures you should use in the above methods are described in Box 57.1. In addition to these methods, the prosthetic groups of conjugated proteins often have characteristic absorption maxima, for example, the haem group of haemoglobin absorbs strongly at 415 nm, which you can use for quantitative assay. Other methods exploit the biological properties of proteins and peptides, for example immunoassay (p. 287), enzymatic analysis and affinity chromatography (pp. 353–5), based on specific biological interactions.

Most assays for peptides and proteins do not give absolute values, but require standard solutions, containing appropriate amounts of a particular protein, to be analysed at the same time, so that a standard curve can be constructed. Bovine serum albumin (BSA) is commonly used as a protein standard. However, you may need an alternative standard if the protein you are assaying has an amino acid composition that is markedly different from that of BSA, depending on your chosen method.

One of the aims of peptide and protein purification is to determine the 3D structure ('native conformation') of these molecules as an aid to understanding their function. The methods commonly used are described below. This information can be used, for example, in understanding certain genetic diseases, or when understanding drug action or studying enzyme structure and function (Chapter 62).

Box 57.1 How to determine the amount of protein/peptide in an aqueous solution

The following methods apply to proteins in aqueous solution, and the amounts indicated are appropriate for semi-micro cuvettes (1.5 mL volume, path length 1 cm). Note that you must prepare and analyse appropriate controls (blanks), to assess possible interference (due to buffers, etc.).

A. Biuret method

This is based on the specific reaction between cupric ions (Cu^{2+}) in alkaline solution and two adjacent peptide bonds, as found in proteins and peptides. As such, it is not significantly affected by differences in amino acid composition.

1. **Prepare protein standards over an appropriate range** (typically, between 1 and 10 mg mL^{-1}).
2. **Add 1 mL of each standard solution to separate test tubes. Prepare a reagent blank, using 1 mL of distilled water, or an appropriate solution.**
3. **Add 1 mL of each unknown solution to separate test tubes.**
4. **Add 1 mL of biuret reagent (1.5 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 6.0 g sodium potassium tartrate in 300 mL of 10 w/v NaOH) to all standard and unknown tubes and to the reagent blank.**
5. **Incubate at 37 °C for 15 min.**
6. **Read the absorbance of each solution at 520 nm against the reagent blank.** The violet colour is stable for several hours.

The main limitation of the biuret method is its lack of sensitivity – it is unsuitable for solutions with a protein content of less than 1 mg mL^{-1} .

B. Direct measurement of UV absorbance (Warburg-Christian method)

Proteins and peptides absorb EMR maximally at 280 nm (owing to the presence of aromatic amino acids) and this forms the basis of the method. The principal advantages of this approach are its simplicity and the fact that the assay is non-destructive. The most common interfering substances are nucleic acids, which can be assessed by measuring the absorbance at 260 nm: a pure solution of protein will have a ratio of absorption (A_{260}/A_{280}) of approximately 0.6, decreasing with increasing nucleic acid contamination. Note also that any free aromatic amino acids in your solution will absorb at 280 nm, leading to an overestimation of protein content. The simplest procedure, which includes a correction for small amounts of nucleic acid, is as follows (use quartz cuvettes throughout).

1. **Measure the absorbance of your solution at 280 nm (A_{280}):**

if A_{280} is greater than 1, dilute by an appropriate amount and remeasure.

2. **Repeat at 260 nm (A_{260}).**
3. **Estimate the approximate protein concentration of the solution using the following relationship:**

$$[\text{protein}] \text{ mg mL}^{-1} = 1.45A_{280} - 0.74A_{260} \quad [57.1]$$

This equation is based on the work of Warburg and Christian (1942) for enolase. For other proteins, it should not be used for quantitative work, since it gives only a rough approximation of the amount present, due to variations in aromatic amino acid composition.

C. Lowry (Folin-Ciocalteu) method

This is a colorimetric assay, based on a combination of the biuret method, described above, and the oxidation of tyrosine and tryptophan residues with Folin-Ciocalteu reagent to give a blue-purple colour. The method is extremely sensitive (down to a protein/peptide content of 20 $\mu\text{g mL}^{-1}$), but is subject to interference from a wide range of non-protein substances, including many organic buffers (e.g. TRIS, HEPES), EDTA, urea and certain sugars. The choice of an appropriate standard is important, as the intensity of colour produced for a particular protein/peptide is dependent on the amount of aromatic amino acids present.

1. **Prepare protein standards within an appropriate range for your samples** (the method can be used from 0.02 to 1.00 mg mL^{-1}).
2. **Add 1 mL of each standard solution to separate test tubes. Prepare a reagent blank, using 1 mL of distilled water, or an appropriate solution.**
3. **Add 1 mL of each of your unknown solutions to separate test tubes.**
4. **Then, add 5 mL of 'alkaline solution' (prepared by mixing 2% w/v Na_2CO_3 in 0.1 mol L^{-1} NaOH, 1% w/v aqueous CuSO_4 and 2% w/v aqueous NaK tartrate in the ratio 100 : 1 : 1. Mix thoroughly and allow to stand for at least 10 min.**
5. **Add 0.5 mL of Folin-Ciocalteu reagent (commercial reagent, diluted 1 : 1 with distilled water on the day of use).**
Mix rapidly and thoroughly and then allow to stand for 30 min.
6. **Read the absorbance of each sample at 600 nm.**

continued

Box 57.1 (continued)**D. Dye-binding (Bradford) method**

Coomassie brilliant blue combines with proteins and peptides to give a dye-protein complex with an absorption maximum of 595 nm. This provides a simple and sensitive means of measuring protein content, with few interferences. However, the formation of dye-protein complex is affected by the number of basic amino acids within a protein, so the choice of an appropriate standard is important. The method is sensitive down to a protein content of approximately $5 \mu\text{g mL}^{-1}$ but the relationship between absorbance and concentration is often non-linear, particularly at high protein content.

1. **Prepare protein standards over an appropriate range (between 5 and $100 \mu\text{g mL}^{-1}$).**
2. **Add $100 \mu\text{L}$ of each standard solution to separate test tubes. Prepare a reagent blank, using $100 \mu\text{L}$ of distilled water, or an appropriate solution** (note that these small volumes must be accurately dispensed, e.g. using a calibrated pipettor, p. 143).
3. **Add $100 \mu\text{L}$ of your unknown solutions to separate test tubes.**
4. **Add 5.0 mL of Coomassie brilliant blue G250 solution (0.1 g L^{-1}).**
5. **Mix and incubate for at least 5 min: read the absorbance of each solution at 595 nm.**

Determining the structure of a protein

The process of establishing a protein's 3D configuration requires accurate knowledge of its primary structure, as outlined below.

Identifying the amino acid content

To determine the primary structure of a purified protein, your first step is to hydrolyse the polypeptide and determine the constituent amino acids. This is usually achieved using $6 \text{ mol L}^{-1} \text{ HCl}$ at 110°C for 24–72 h; note that you must take great care when working with hot acids. You can separate and tentatively identify the amino acids by several techniques, including thin-layer chromatography and high-performance liquid chromatography (Chapter 48). For advanced studies a dedicated amino acid analyser may be used; a polystyrene resin-based cation exchange column is used to separate the amino acids on the basis of ion-exchange and hydrophobic interactions.

Analysing the amino acid sequence

Having obtained the amino acid composition of the protein, your next step is to determine the order of the amino acid residues along the polypeptide chain (Fig. 57.3(a)). You can achieve this by the following process.

1. **Cleaving the polypeptide** at specific peptide bonds, resulting in smaller fragments (peptides); for example, using cyanogen bromide (CNBr), which cleaves peptide bonds formed by the carboxyl group of met, or by proteolytic enzymes, for example, trypsin (cleaving after lys and arg residues) and chymotrypsin (cleaving after phe, tyr and trp).
2. **Separating the peptides**, for example, by column chromatography.
3. **Determining the sequence of each peptide** by a process called Edman degradation. This involves the selective removal of the N-terminal amino acid by treating the fragments with phenyl isothiocyanate ($\text{C}_6\text{H}_5\text{N}=\text{C}=\text{S}$) followed by acid hydrolysis; the substituted phenylthiohydantoin formed can then be identified by chromatography. The process can be repeated as many as 50 times, releasing the N-terminal residue on each occasion, and allowing the sequence of each fragment to be established.

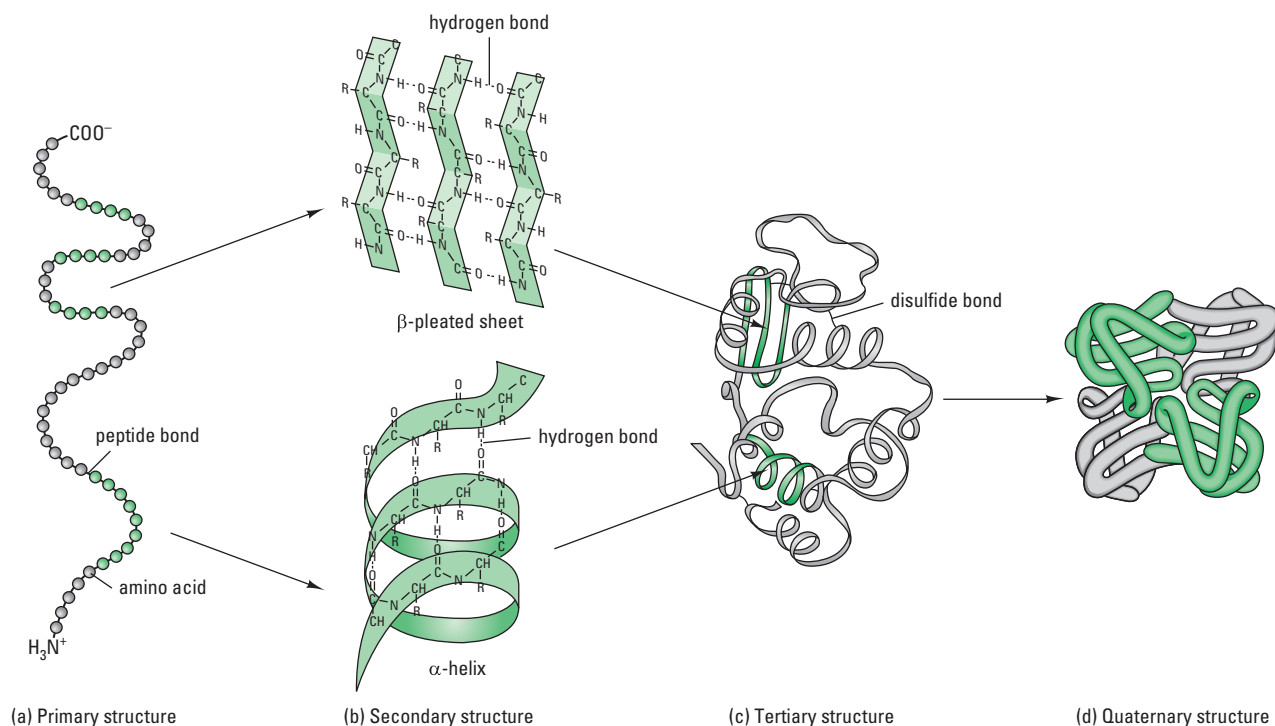


Fig. 57.3 Representation of the elements of the three-dimensional conformation of peptides and proteins. Adapted from Enciso (2012). Note that only certain portions of the primary structure may exist in the secondary structures shown, and that the latter may not appear in every tertiary structure.

4. Matching the sequenced, overlapping peptide fragments, to determine the overall sequence of amino acid residues in the original protein.

Determining the secondary, tertiary and quaternary structure

Taking account of modifications to protein structure' and continuing – As part of their functionality, proteins may undergo modifications involving further covalent reactions. Examples include phosphorylation (generally of ser, thr or tyr residues), glycosylation, and ubiquitination. Co-factors may also associated with proteins to aid function; these include ions (e.g. $\text{Fe}^{2+}/\text{Fe}^{3+}$ in the haem group of haemoglobin) and prosthetic groups such as vitamin derivatives (e.g. biotin in various carboxylation enzymes).

While the 3D structure of proteins can be investigated by X-ray analysis of crystallised purified proteins, this would more generally be achieved by using computer-based molecular modelling programs that predict the 3D conformation of a protein from information based on the primary structure (Chapter 70). These take account of the likelihood of hydrogen bonding and covalent bonding within the structure. Thus, the make-up of the primary structure is assessed to determine bond angles, residue interactions and the potential for sections of the chain to form secondary structures such as β -pleated sheets and α -helices (Fig. 57.3(b)) which may be incorporated into the tertiary structure. The tertiary structure is typically stabilised by hydrogen bonding and covalent bonds (e.g. disulfide bonds between spatially close cys residues). Taking account of all these possibilities leads to a 3D representation of the molecule (Fig. 57.3(c)). Some proteins (e.g. haemoglobin) are assembled in multiple units, providing a quaternary structure (Fig. 57.3(d)).

Text references

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STUDY EXERCISES

57.1 Test your knowledge of the basis of protein assays.

Match up each of the following methods with the corresponding underlying reaction: Method: (a) Warburg–Christian; (b) Bradford; (c) Lowry (Folin–Ciocalteu); (d) Biuret.

Reaction: (i) Coomassie blue binding; (ii) copper(II) ion–peptide bond interaction; (iii) absorption of UV radiation by aromatic amino acids; (iv) a combination of the copper(II) ion–peptide bond reaction and the oxidation of tyrosine and tryptophan residues.

57.2 Determine the amount of amino acid in samples, using a 'standard curve' (see also study exercise 42.2 for an equivalent exercise for protein assay).

The data in the following Table are for a set of calibration standards, measured using the ninhydrin method, which is non-linear at high amino acid concentrations.

- Draw a calibration curve by hand using graph paper and estimate the concentration of amino acid in three different test samples giving the following A_{570} values: (i) 0.341; (ii) 0.686; (iii) 0.969.
- Assuming that these A_{570} values were obtained using the following dilutions and sample volumes

Absorbance of calibration standards.

Amino acid concentration in assay tube ($\mu\text{g mL}^{-1}$) (total assay volume 5 mL)	Absorbance of standard (A_{570})
0.0	0.000
2.0	0.151
4.0	0.305
6.0	0.481
8.0	0.623
10.0	0.751
12.0	0.866
14.0	0.935
16.0	0.977
18.0	1.012
20.0	1.028

in a total assay volume of 5 mL for all samples and standards alike, calculate the amino acid concentration of each of the three original samples, consisting of: (i) 100 μL of undiluted sample; (ii) 50 μL of a 10-fold dilution of sample; (iii) 80 μL of a 25-fold dilution of sample.

Give all answers in mg mL^{-1} , to one decimal place.

(continued)

57.3 Determine the amino acid sequence of a polypeptide from cleavage fragment data.

The following data represent the results for a polypeptide of unknown amino acid sequence, when a subsample of the polypeptide was digested to completion with trypsin while another subsample was cleaved with cyanogen bromide (CNBr). In both cases, four peptide fragments were produced. These peptide fragments produced were then subjected to Edman degradation, and the

following results were obtained for each peptide, in order of successive rounds of Edman degradation.

Tryptic digestion products: fragment (i): val asn lys; fragment (ii): ala gly met ser arg; fragment (iii): trp phe met ala ala; fragment (iv): his gly met ala glu lys.

CNBr cleavage products: fragment (i): ala ala; fragment (ii): his gly met; fragment (iii): ser arg trp phe met; fragment (iv): ala glu lys val asn lys ala gly met. What is the sequence of the intact polypeptide?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

58 Assaying lipids

Table 58.1 Some examples of fatty acids.

No. of carbon atoms	Systematic name	Trivial name
Saturated fatty acids (no C=C bonds)		
12	<i>n</i> -Dodecanoic	Lauric
14	<i>n</i> -Tetradecanoic	Myristic
16	<i>n</i> -Hexadecanoic	Palmitic
18	<i>n</i> -Octadecanoic	Stearic
20	<i>n</i> -Eicosanoic	Arachidic
22	<i>n</i> -Docosanoic	Behenic
Mono-unsaturated fatty acids (one C=C bond)		
12	<i>cis</i> -9-Dodecenoic	Lauroleic
14	<i>cis</i> -9-Tetradecenoic	Myristoleic
16	<i>cis</i> -9-Hexadecenoic	Palmitoleic
18	<i>cis</i> -9-Octadecenoic	Oleic
20	<i>cis</i> -9-Eicosenoic	Gadoleic
22	<i>cis</i> -9-Docosenoic	Erucic

Note: palmitic, stearic, palmitoleic and oleic acids are quantitatively the most common fatty acids in the majority of organisms. Major polyunsaturated fatty acids include linoleic acid (C₁₈, two double bonds), linolenic acid (C₁₈, three double bonds) and arachidonic acid (C₂₀, four double bonds).

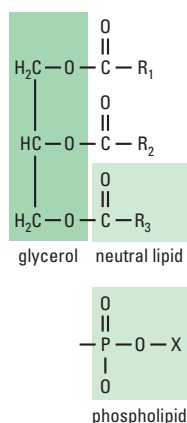


Fig. 58.1 General structure of lipids. In neutral lipids, the triacylglycerol component (darker shaded box) is esterified to three fatty acids R_x (Table 58.1). In a phospholipid, the triacylglycerol component is the same but the end esterified group (lighter shaded box) is substituted by a phosphoryl group as shown immediately below. Here, X = a hydrophilic group, for example, $-\text{CH}_2\text{CH}_2\text{NH}_3^+$ in phosphatidyl ethanolamine and $-\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3^+$ in phosphatidyl choline.

The term ‘lipid’ is used to describe a broad group of compounds with a wide variety of chemical structures, physical properties and biological functions. Biological lipids are often subdivided into two main types, simple and complex, each of which contains one or more fatty acids as a major structural component. This leads to their hydrophobic nature and hence insolubility in water – a defining feature. Lipids in biological systems may be combined with either proteins (for example, in lipoproteins) or polysaccharides (for example, in lipopolysaccharides).

Lipids perform important roles in all living organisms, including:

- **structural components of biological membranes**, for example, phospholipids, glycolipids, and cholesterol
- **energy storage**, for example, oils such as triacylglycerols in plant seeds or the lipid polyhydroxyalkanoate in bacterial storage granules
- **insulation**, for example, subcutaneous triglyceride fat deposits in mammals such as whales
- **waterproofing**, for example, as a component of waxes in the cuticles of plant leaves
- **cell-cell recognition and cell-cell signalling**, for example, glycosphingolipids are determinants of human ABO blood groups, while sphingolipids are involved in cell–cell recognition.

KEY POINT Because of their diversity and complexity, lipids are often referred to by more than one name (Table 58.1), and the various types of non-systematic names can be confusing.

Understanding the structure and properties of lipids

Simple or neutral lipids

Neutral fats (triglycerides or triacylglycerol) are esters of fatty acids and glycerol, as shown in Fig. 58.1. Fatty acids are straight-chain carboxylic acids, typically with an even number of carbon atoms and chain lengths of C₁₂–C₂₂, which may be saturated or unsaturated (Table 58.1). The greater the chain length and the fewer the number of double bonds, the higher the melting point, making most long chain saturated fatty acids solids at room temperature. Glycerol is the most common alcohol found in simple lipids, though higher M_r alcohols occur in waxes, and cyclic alcohols (sterols, for example, cholesterol) occur in bile acids, steroid hormones and vitamins (for example, vitamin K). While glycerol is a liquid at room temperature, cholesterol remains solid up to 150 °C.

The major categories of simple lipids are:

- **fats** – for example, animal triglycerides that contain mainly saturated fatty acids (Table 58.1) and are solids at room temperature
- **oils** – for example, plant triglycerides (‘oils’) that have relatively short chain lengths, a greater degree of unsaturation and are liquids at room temperature

- **waxes** – esters of fatty acids with alcohols of higher M_r than glycerol. Usually solid at room temperature
- **cyclic alcohols** – for example, sterols such as cholesterol. These occur in bile acids, as steroid hormones and vitamins (for example, vitamin K).

Complex, compound or polar lipids

These are acyl esters of glycerol, or the amino alcohol sphingosine, that also include a hydrophilic group (for example, a phosphoryl or carbohydrate group). The major types of complex lipid are:

- **Phospholipids** – the most common forms (phosphoglycerides) are based on phosphatidic acid, with two fatty acids esterified to glycerol. Most phospholipids also contain a hydrophilic amino alcohol or a similar group, attached to the phosphoryl group (Fig. 58.1). The principal phospholipid classes are: (i) phosphatidyl cholines (or lecithins), which form stable emulsions with water and dissolve completely on addition of bile salts – these are insoluble in acetone, a feature that enables lecithins to be separated from most other lipids; (ii) phosphatidyl ethanolamines (or cephalins) – unlike lecithins, these are insoluble in ethanol and methanol; (iii) phosphatidyl serine; (iv) phosphatidyl inositol; and (v) plasmalogens.
- **Sphingolipids** – these incorporate the amino dialcohol, sphingosine, rather than glycerol (Fig. 58.2). Fatty acids are linked to sphingosine via an amide bond to form ceramides, which include: (i) cerebrosides (glycosphingolipids); (ii) sulfatides (sulfated cerebrosides); (iii) gangliosides (glycosphingolipids containing sialic acid residues); and (iv) phosphosphingolipids, including sphingomyelins, which are esters of a ceramide and phosphoryl choline.

KEY POINT An important feature of complex lipids is their amphipathic nature, i.e. each molecule has a polar (hydrophilic) and a non-polar (hydrophobic) region. This property enables phospholipids to form bilayers in biological membranes.

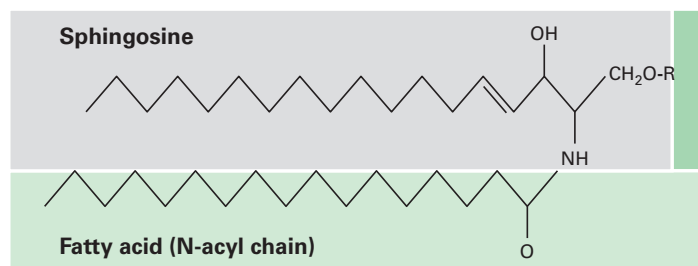


Fig. 58.2 General structure of a sphingolipid. Sphingosine is an unsaturated 18-C amino alcohol. The attached fatty acid component can be of a variety of chain lengths. If $R = H$, then the molecule is classed as a ceramide; if $R =$ phosphocholine, as a sphingomyelin; and if $R =$ a carbohydrate, as a cerebroside or a ganglioside.

Partitioning of aqueous and organic phases in extraction – you would normally carry this out using a separating funnel.

SAFETY NOTE Working with solvents – chloroform and benzene must be used with care owing to their high toxicity; they are often replaced by dichloromethane and toluene respectively. All mixing and pouring steps should be carried out in a spark-free fume cupboard. Note that ethers may form explosive peroxides on prolonged storage. Lipid extracts extracted in flammable solvents must be stored in a spark-proof refrigerator, not in routine lab fridges.

Definition

Emulsion – a colloidal mixture where one liquid is dispersed (but not dissolved) in another liquid, e.g. lipids often aggregate to form micelles in aqueous solutions.

Definition

Sulfolipid – lipids which contain a sulfur-containing functional group. These are components in the thylakoid membranes of chloroplasts, for example.

Extracting and separating lipids

Extraction from cells and tissues

The relative insolubility of lipids in water means that you can only extract them from biological material using organic solvents such as acetone, ether and chloroform. You would normally use a homogeniser beforehand to disrupt cell and tissue structure (Chapter 42). You can select between two possible approaches:

- 1. Total extraction of all lipids, followed by separation of the different lipid classes.** A commonly used solvent mixture is methanol : chloroform : water (2 : 1 : 0.5, v/v/v). When you add an equal volume of aqueous 1% w/v NaCl solution to the extract, this results in the formation of two immiscible layers: the lipids are present in the lower layer, while the upper aqueous layer contains other biomolecules (for example, proteins). You can also use hexane : isopropanol (3 : 2, v/v) instead of chloroform : methanol – it is less toxic and it dissolves very little non-lipid material.
- 2. Selective lipid extraction.** You can extract neutral lipids (for example, those in storage tissues) from the aqueous phase by partitioning against relatively non-polar solvents including hexane, diethyl ether and chloroform. If you wish to extract membrane lipids like phospholipids, this requires disruption of the membrane using more polar solvents such as methanol or ethanol, followed by selective precipitation by adding cold, dry acetone. You can extract glycolipids using acetone.

Problems associated with solvent extraction methods include: (i) the requirement for large volumes of potentially hazardous solvents; and (ii) the possible formation of an emulsion, leading to incomplete extraction of component lipids. You will therefore need to use appropriate safety procedures, while you can reduce the risk of emulsion formation by less vigorous mixing of phases (i.e. swirling rather than shaking), by acidifying the medium or by adding NaCl or methanol.

Adsorption chromatography

Here, you use silica gel, octadecylsilane-bonded silica or ion-exchange resins to bind solvent-extracted lipids by a combination of polar, ionic and van der Waals forces. In practice, a glass column is packed with a slurry of adsorbent in an appropriate organic solvent, and the lipid extract (dissolved in the same solvent) is applied to the top of the column. You can then selectively elute lipids: a mixture can be broadly separated into neutral lipids, glycolipids and phospholipids using solvents of increasing polarity, for example, chloroform → acetone → methanol. You can further separate the lipid sub-fractions on silica gel columns, as follows:

- **neutral lipids** can be separated and eluted using hexane containing increasing proportions of diethyl ether (0 → 100% v/v) in the order: hydrocarbons, cholesterol esters, triacylglycerols, free fatty acids, cholesterol, diacylglycerols, then monoacylglycerols
- **glycolipids and sulfolipids** can be separated by first eluting the glycolipids with chloroform : acetone (1 : 1 v/v), then using acetone to elute the sulfolipids

Preventing oxidative rancidity – all lipids will oxidise (become rancid) when exposed to air in daylight, due to hydrolysis and/or photo-oxidation. Keep extracted lipids in the dark, and add an antioxidant such as butylated hydroxytoluene for longer term storage. Minimise oxidation by flushing vessels with nitrogen gas and bubbling solvents with (oxygen-free) nitrogen during analysis.

Understanding silica gel codes – silica gel is manufactured as particles of 10–15 μm diameter, containing pores of diameter 40, 60, 80, 100 or 150 Å (Ångstrom, p. 194), where Å = 10^{-10} m (thus, silica gel 60 has a pore size of 60 Å). Silica gel G contains calcium sulfate to assist binding to the glass support, while silica gel H has no binder.

- **phospholipids** can be eluted using chloroform containing increasing proportions of methanol (5% → 50% v/v) in the order: phosphatidic acid, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, phosphatidyl inositol, sphingomyelin.

Thin-layer chromatography (TLC)

You can use this technique to separate lipid mixtures into their constituents, or to quantify particular lipids, as part of an analytical procedure. The principle of the technique is described in Chapter 48. Silica gel G60 is the most frequently used stationary phase, acting as a polar absorbent. When you carry out separation with a non-polar mobile phase, non-polar lipids will migrate more rapidly (i.e. they will have high R_F values, p. 347), while polar lipids will migrate more slowly. By increasing the polarity of the solvent, the R_F values of the polar lipids can be increased. Your choice of solvent will depend on the lipids in the extract:

- **For neutral lipids**, a typical solvent system is hexane : diethyl ether : glacial acetic acid at 80 : 20 : 2 (v/v/v), separating in the following order of decreasing R_F : steryl esters, wax esters, fatty acids, methyl esters, triacylglycerols, fatty acids, fatty alcohols, sterols, 1,2-diacylglycerols, monoacylglycerols.
- **For polar lipids**, silica gel H is preferred, as silica gel G prevents the separation of acidic phospholipids. Most solvent systems for polar lipids are based on chloroform : methanol : water, for example, at 65 : 25 : 4 (v/v/v). In this system, the relative order of migration is: mono-galactosyldiacylglycerol, cerebrosides, phosphatidic acid, cardiolipin, lysophosphatidyl ethanol-amine, phosphatidyl ethanolamine and digalactosyldiacylglycerol, sulfatides, phosphatidyl choline, phosphatidyl inositol, sphingomyelin and phosphatidyl serine.

KEY POINT No single solvent system will completely separate all lipid components in a single TLC procedure. However, 2D TLC (pp. 347–8) may separate up to 200 individual lipid constituents from a sample.

You can also separate lipids by employing reversed-phase TLC (RP-TLC), where the silica gel is made non-polar (for example, by silanisation), and highly polar solvents are used as the mobile phase: here, the polar lipids will have the highest R_F values and the non-polar lipids the lowest R_F values.

Detecting and quantifying lipids and their components

You can detect lipids in the R_F zones of TLC plates by staining. You can choose to immerse the plate in the stain, or the stain can be sprayed. The stains are generally non-specific, and methods include:

- **spraying with iodine solution** (for example, 1–3% w/v in chloroform) – most lipids appear as brown spots on a yellow background, though glycolipids stain weakly by this method
- **treatment with a strong oxidising agent followed by charring** (for example, spray with 5% v/v sulfuric acid in ethanol, followed by heating in an oven at 180 °C for 30–60 min) – lipids appear as black deposits; the detection limit is 1–2 μg

SAFETY NOTE Lipid stains often contain corrosive or toxic reagents, so all manipulations should be performed in a fume cupboard or a commercial spray cabinet. When spraying TLC plates with sulfuric acid solutions, this must be carried out on a suitable support (e.g. disposable paper or card) within the fume hood. Wear safety glasses and gloves throughout the procedure.

Quantifying lipids separated by TLC – you can use scanning densitometry to provide quantitative information; however, it is better to have more molecule-specific information than an R_f value if possible as this parameter alone might characterise many possible compounds.

Definitions

Iodine number – the amount of iodine (g) absorbed by 100 g of fat, owing to the reaction of iodine with C=C bonds within the fat. Typically within the range 30–200.

Saponification – the hydrolysis of an ester under alkaline conditions, to form an alcohol and the salt of the acid.

Saponification value – the amount of KOH (in mg) required to saponify 1 g of fat completely. Typically within the range 150–300.

Example A 0.15-g sample of cod liver oil was titrated against $0.1 \text{ mol L}^{-1} \text{ Na}_2\text{S}_2\text{O}_3$, giving a titration volume of 31.7 mL (V_t), compared with a blank of 49.8 mL (V_o). Substituting into eqn [58.1] gives an iodine number of $[1.27(49.8 - 31.7)] \div 0.15 = 153$ (to three significant figures).

- **fluorescent stains** (for example, the widely used 2',7'-dichlorofluorescein, DCF, at 0.1–0.2% w/v in ethanol). Under UV, lipids appear as bright yellow spots on a yellow–green background; the limit of detection is 5 µg. Other fluorescent stains, such as 1-anilo-8-naphthalene sulfonate (ANS, 0.1% w/v in water), can be used to detect nanogram quantities of lipid.

Saponification and iodination

While it is possible to use TLC staining to quantify particular lipids, you would more commonly assay the compounds released on hydrolysis of simple or complex lipids, namely the alcohols, fatty acids or other components, rather than the native lipid. Alkali hydrolysis of lipids containing fatty acids results in the formation of a soap (i.e. saponification): for example, the incubation of tripalmitin with KOH yields potassium palmitate and glycerol. Acid hydrolysis of triacylglycerols releases 'free' fatty acids and glycerol.

You can gain basic information about the relative size of the fatty acid component of oils and fats from the saponification value, determined by titration against $0.8 \text{ mol L}^{-1} \text{ KOH}$; the lower the saponification value, the higher the M_r of the fatty acids. The degree of unsaturation of the fatty acids is given by the iodine number; the higher the iodine number, the greater the content of unsaturated fatty acids.

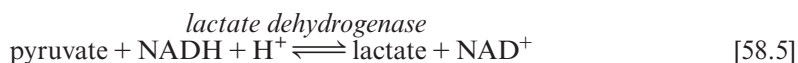
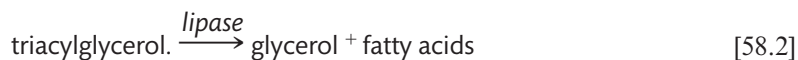
To obtain the iodine number for a particular fat, titrate the free iodine remaining after reaction against 0.1 mol L^{-1} sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) using a trace amount of starch as an indicator, giving a titration volume for the test solution, V_t . A blank containing no fat is also titrated, to establish the volume of sodium thiosulfate required to titrate the initial free iodine, V_o . This allows the amount of iodine that reacts with the fat to be calculated according to the formula:

$$\text{iodine number (in g)} = \frac{1.27(V_o - V_t)}{m} \quad [58.1]$$

where m is the mass of test fat (in g), and V_o and V_t are expressed in millilitres.

Measurement of glycerol content

You can quantify glycerolipids by measuring the glycerol released on hydrolysis. A widely used method involves a coupled enzyme assay (pp. 460–1), with the following reactions:



The glycerol concentration is determined by measuring the decrease in absorbance of NADH at 340 nm (A_{340}), compared to that of a blank with no added lipase.

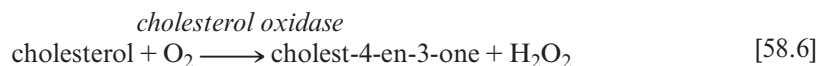
Understanding coupled enzyme assays based on NADH/NAD⁺ interconversion – these are explained in more detail on p. 436, while the procedure required to convert changes in A_{340} to [NADH] is given on p. 460.

Using alternative approaches for measuring H₂O₂ – the hydrogen peroxide produced from the oxidation of cholesterol in eqn [58.6] can be converted to water and oxygen by the enzyme catalase ($2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$) and the oxygen produced can be quantified, for example, using an oxygen electrode (pp. 385–6).

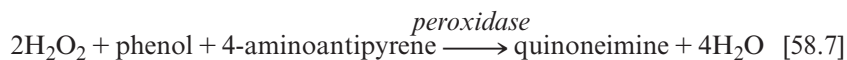
Example For gas chromatography, you can derivatise fatty acids to their methyl esters by boiling for 2–3 min with boron trifluoride (BF₃) solution (14%w/v in methanol) prior to analysis to increase their volatility and stability in the gas phase. The separated components are normally detected by flame ionisation (p. 359).

Measurement of cholesterol content

You can measure the cholesterol in cholesterol esters after hydrolysing to free cholesterol using cholesterol esterase. The subsequent assay is as follows:



The hydrogen peroxide produced as a result of the action of cholesterol oxidase can be measured amperometrically or colorimetrically, via the peroxidase-catalysed reaction:



This is the basis of many commercially available cholesterol testing kits. An alternative approach is to measure the cholest-4-en-3-one directly, *via* its absorbance maximum at 240 nm.

Gas chromatography (GC) of lipids and lipid components

You can use this technique for the qualitative and quantitative analysis of a broad range of lipids. Volatile lipids may be analysed without modification, while non-volatile lipids must first be converted to a more volatile form, either by degradation (for example, phospholipids), or derivatisation (p. 358). The retention time in GC depends on each molecule's volatility: those lipids with shorter hydrocarbon chains will be more volatile at the high temperature used and will therefore elute first. There is an approximately linear relationship between log retention time and the number of carbon atoms in the lipid molecule. Figure 58.3 shows a representative GC trace for a sample with a mix of lipid compounds.

The most effective GC columns are support-coated open tubular (SCOT) capillary columns, with a thin film (0.1–10 μm) of the stationary phase coated onto the internal wall. Your choice of stationary phase depends on

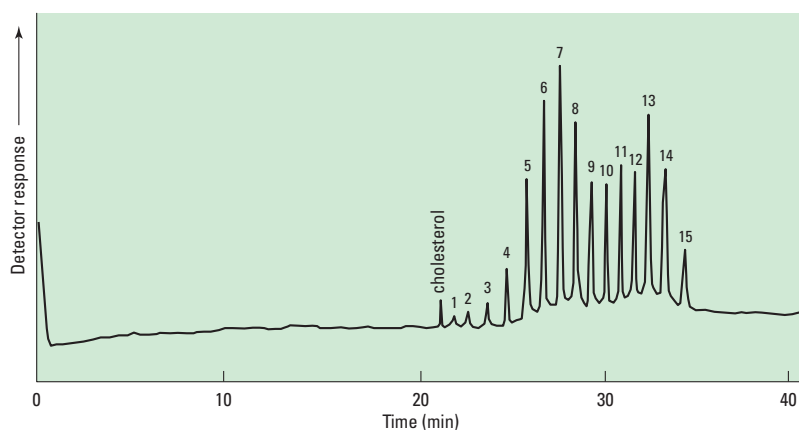


Fig. 58.3 Representative GC trace demonstrating the analysis of a lipid mixture. This chromatogram shows the results of GC-FID (Chapters 48–49) of triacylglycerols in butter fat. The lower M_r molecules, with greater volatility, are the ones eluting first. There are 15 peaks, corresponding to lipids with even numbers of acyl carbon atoms between 26 and 54. In such studies, the identity of each peak is determined from coincident retention times for standards.

Using advanced methods for identifying lipids – although not routinely available to students, methods such as HPLC (Chapters 48 and 49) and mass spectrometry (Chapter 47) are also used in research labs to detect and quantify lipids (see Christie and Han, 2016).

the components to be separated; for example, some non-polar stationary phases cannot resolve methyl esters of saturated and monounsaturated fatty acids. Many stationary phases are based on silicone greases or polysiloxane, ranging from the non-polar dimethyl polysiloxanes (for example, OV-101) to the polar trifluoropropyl methyl polysiloxanes (for example, OV-210). Other polar stationary phases are based on polyethylene glycol (for example, Carbowax 20M).

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STUDY EXERCISES

58.1 Consider how to avoid oxidative rancidity. What practical steps could you take to avoid lipid oxidation during extraction and analysis?

58.2 Use the web to discover more about lipid structure and analysis. Visit the Cyberlipid Center website (at <http://cyberlipid.gerli.com/>), a resource giving details of a wide range of lipid structures, including triglycerides, phospholipids and glycolipids: (a) identify the main lipid components of beeswax and (b) suggest which separation methods might be appropriate for the analysis of these components.

58.3 Derive the equation for calculation of the iodine number of a lipid. Given that the following reactions (i)–(iii) are involved in the volumetric analysis for calculating the iodine number of a lipid, show how Eqn [58.1] is derived:

- (i). $-\text{CH}=\text{CH}- + \text{ICl} \rightarrow -(\text{I})\text{CH}-\text{CH}(\text{Cl})-$
- (ii). $\text{ICl} + \text{KI} \rightarrow \text{HC1} + \text{I}_2$
- (iii). $\text{I}_2 + 2\text{Na}_2\text{S}_2\text{O}_3 \rightarrow 2\text{NaI} + \text{Na}_2\text{S}_4\text{O}_6$

Answers to these study exercises are available at go.pearson.com/uk/he/resources

59 Assaying carbohydrates

These are compounds with a formula based on $C_x(H_2O)_x$. They may exist individually or in polymeric forms (Table 59.1) and can also be present as heteropolymers, for example, when linked to protein in glycoproteins (where carbohydrates form the minor component), or proteoglycans (where they form the major component). Table 59.1 describes the key features and main types of carbohydrate, together with examples of typical biological roles.

KEY POINT The identification and quantitative analysis of carbohydrates in biological samples can be difficult, owing to structural and physicochemical similarities between related compounds. Several routine analytical methods cannot distinguish between isomeric forms of a particular carbohydrate.

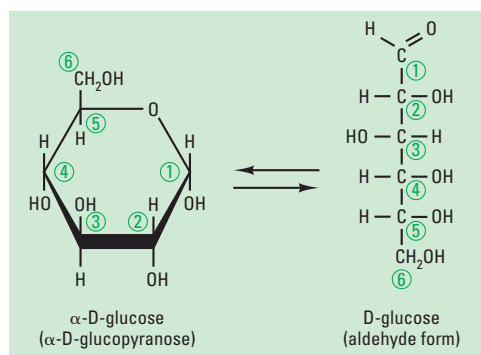


Fig. 59.1 Ring and straight chain forms of glucose. Note that β -D-glucose has H and OH groups reversed at C-1 in the pyranose form.

Understanding the structure and properties of carbohydrates

Monosaccharides

The simplest carbohydrates are the monosaccharide sugars, which are polyhydroxy aldehydes and polyhydroxy ketones (so-called aldoses and ketoses), typically with three to seven carbon atoms per molecule. The common names end with the suffix ‘-ose’, for example, glucose (Fig. 59.1), which contains six carbon atoms and is a hexose. The simplest aldose is the three-carbon compound (triose) glyceraldehyde (Fig. 59.2(a)), and you can consider the other aldoses to be derived from glyceraldehydes, by the addition of successive secondary alcohol groups ($H-C-OH$). In a similar

Table 59.1 A summary of the main types of biologically important carbohydrates. After Clemens *et al.* (2016).

Type	Monosaccharides	Disaccharides	Oligosaccharides	Polysaccharides	Heteropolymers
Key features	Single sugar molecule; usually sweet-tasting	Two sugar molecules; usually sweet-tasting	3–10 sugar molecules; not sweet in native form	10+ sugar molecules; not sweet in native form	Sugar linked to protein; often biologically active
Examples	Glucose Fructose Galactose Mannose	Sucrose Lactose Maltose	Digestible Maltotriose Maltotetrose Maltopentose Non-digestible Raffinose Stachyose	Digestible Linear: Starch Branched: Glycogen Non-digestible (dietary fibre) Soluble: agar, gum arabic, pectin Non-soluble: cellulose, chitin	As minor component (glycoproteins) Mucins Immunoglobulins As major component (proteoglycans) Chondroitins Fibromodulin
Biological roles (examples)	Energy storage and transport; pollinator attraction	Energy storage and transport; Energy storage; pollinator attraction	Energy storage; markers of cellular identity; cell binding	Energy storage; structural components	Immune system (various roles); component of mucus; definer of blood type; component of cellular matrix; ion binding and transport

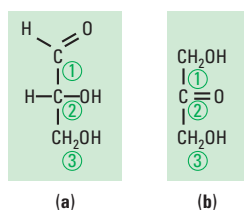


Fig. 59.2 Structure of (a) glyceraldehyde and (b) dihydroxyacetone.

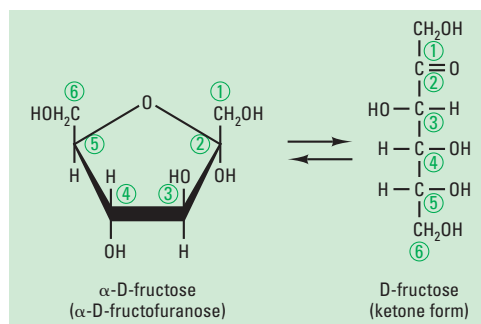


Fig. 59.3 Ring and straight chain forms of fructose. Note that β -D-fructose has OH and CH_2OH groups reversed at C-2 in the furanose form.

Studying the biological roles of

glycosides – these compounds are often formed in plants during the detoxification of certain compounds, or to control plant hormone activity.

manner, you can consider all ketoses to be structurally related to the triose dihydroxyacetone (Fig. 59.2(b)).

Monosaccharides are assigned as D or L isomers (p. 423) according to a convention based on D-glyceraldehyde as the reference compound, with the carbon atoms numbered from the end of the chain containing the reactive group. Most of the carbohydrates found in biological systems are D isomers. While trioses and tetroses exist in linear form, you can represent pentoses and larger monosaccharides either as a linear structure (Fischer form) or as a ring structure (Haworth form), as in Fig. 59.1. The cyclic structure results from the reaction of the carbonyl group (aldehyde or ketone) at one end of the molecule with a hydroxyl group at the other end of the chain, forming a hemiacetal or hemiketal, as shown for glucose (Fig. 59.1) and fructose (Fig. 59.3).

The formation of a hemiacetal or hemiketal creates another asymmetric carbon atom, so that two ring forms exist – one with the $-\text{OH}$ group on this asymmetric carbon positioned below the plane of the ring (α) and another with the $-\text{OH}$ group above the plane of the ring (β). These different isomeric forms are called anomers and are particularly difficult to separate by chromatographic methods. The six-membered ring structure shown for glucose has a structure similar to that of pyran and is termed glucopyranose. A few monosaccharides have a five-membered ring structure similar to that of furan, for example, fructofuranose.

The open chain form is present in very small amounts in aqueous solution: for example, about 0.002% of D-glucose molecules will be in open chain form at 31 °C. However, in this form it has a very reactive carbonyl group which is responsible for the reducing properties of sugars and several analytical methods are based on this feature.

Glycosides

These are formed when a covalent bond, or glycosidic link, is created between the hemiacetal or hemiketal group of a carbohydrate (for example, a monosaccharide) and the hydroxyl group of a second compound (for example, a polyhydroxy alcohol, or another monosaccharide). If the sugars are not joined via their reactive groups, they will still show reducing properties, as in the disaccharide maltose (Fig. 59.4). In contrast, sucrose is a non-reducing disaccharide, since the hemiacetal and hemiketal groups of glucose and fructose are involved in the formation of the glycosidic link.

Polymeric carbohydrates have important biological roles (Table 59.1). The structure of polysaccharides is not fully defined, in contrast to proteins (Chapter 57) and nucleic acids (Chapter 60). This is because polysaccharide structure is the result of the separate actions of a number of biosynthetic enzymes, producing a range of molecules that may vary in both the number of monosaccharide residues and the types of glycosidic bond. The terminology used to describe the glycosidic links in such compounds denotes the anomer involved (α or β) and the C atoms involved in the link, for example, α (1 \rightarrow 4) in maltose (Fig. 59.4), β (1 \rightarrow 4) in cellulose (Fig. 59.5).

Extracting and separating carbohydrates

Most low M_r carbohydrates are soluble in water, however, ethanol : water (80% v/v) is more often used, since polysaccharides and other biological macromolecules are insoluble in aqueous ethanol, allowing you to separate these compounds out. In contrast, polysaccharides are more diverse, and the

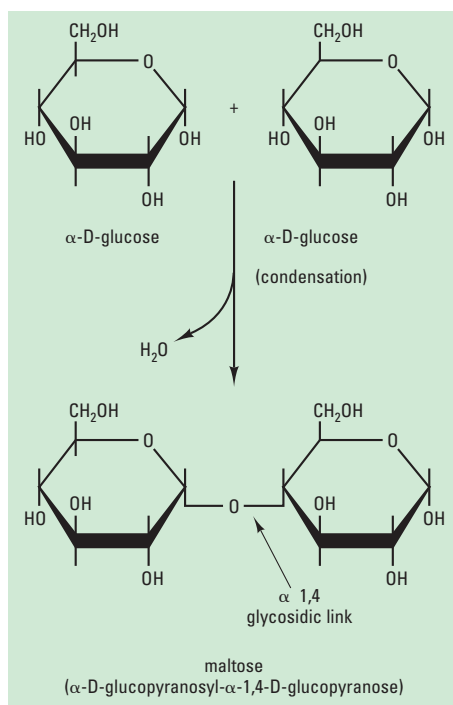


Fig. 59.4 Formation of a glycosidic link ($\alpha(1 \rightarrow 4)$) in the disaccharide maltose.

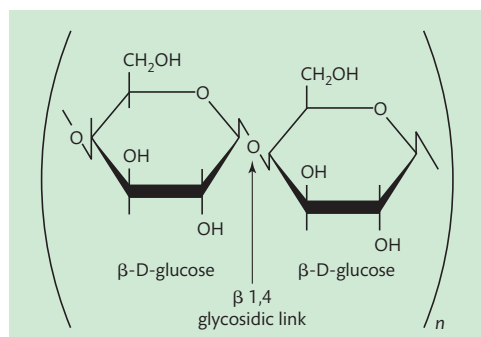


Fig. 59.5 The repeating structure of cellulose ($\beta(1 \rightarrow 4)$ glycosidic links), $n \approx 5000$.

SAFETY NOTE Using the anthrone method – note that this involves hot H_2SO_4 – wear gloves, carry out the heating step in a fume hood and rinse all spillages thoroughly with excess water.

Applying glycosidase assays – some microbial identification schemes are based on the detection of specific glycosidase enzymes, e.g. β -glucuronidase for *E. coli* (p. 462).

isolation procedures vary greatly, for example, boiling water, mild acid or mild alkali can be used to solubilise storage polysaccharides, such as starch, while more vigorous treatment is required for structural polysaccharides: for example, 24% w/v KOH would be used for cellulose. Among the techniques you can use to purify extracted polysaccharides are gel permeation chromatography (pp. 453, 457) and ultracentrifugation (p. 320).

Detecting and quantifying carbohydrates

Chemical methods

Several monosaccharide assay methods are based on the reductive capacity of the aldehyde or ketone groups (Table 59.2). A widely used method for quantitative analysis is that based on reduction of alkaline 3,5-dinitrosalicylate (DNS). This reagent turns from yellow to orange in the presence of reducing sugars and you can measure the absorbance change using a spectrophotometer. The method is also suitable for glycosides, provided the reducing carbonyl groups are not involved in the glycosidic links. Note that it has been reported that some natural furfuryl compounds may interfere with this assay.

Certain polysaccharides react with iodine in acid solution to form coloured complexes: starch gives a blue colour, while glycogen gives a red–brown colour. The anthrone method (typically using 0.1 g L^{-1} anthrone, a tricyclic aromatic ketone, in H_2SO_4 , at 100°C for 10 min, then assayed at 630 nm) is an alternative assay for estimating total carbohydrate content.

You should choose calibration standards carefully for quantitative work – try to use a standard that matches the likely composition of the samples. Note that while such chemical methods can give you a general indication of the relative *amount* of carbohydrate in a sample, they provide you with little useful information on the *types* of carbohydrate present.

Enzymatic methods

These offer a higher degree of specificity in monosaccharide assay, and may even allow you to differentiate between the various stereoisomeric and anomeric forms. The glucose-specific method based on glucose oxidase shown in Table 59.2 also forms the basis of the glucose electrode (see Chapter 52). You can measure the hydrogen peroxide formed using peroxidase and a suitable chromogenic substrate, or by electrochemical methods, for example an amperometric sensor. Alternatively, you can quantify the consumption of oxygen in the initial reaction with an oxygen electrode (pp. 385, 386). Glycosidases can be used to hydrolyse specific disaccharides or polysaccharides into their constituent monosaccharides, which can then be identified and quantified. An example is α -glucosidase, which hydrolyses the $\alpha(1 \rightarrow 4)$ linkage between the glucose residues in maltose (Fig. 59.4). However, you should note that it is rare for such enzymes to show absolute specificity for a particular substrate, so you should be alert to the possibility of interference owing to related compounds in the sample, or to impurities in the enzyme preparation.

Chromatographic methods

Traditional methods include paper and thin-layer chromatographic (TLC) procedures. Suitable supports for TLC include microcrystalline cellulose (in which the sugars partition between the mobile phase and the cellulose-bound water complex) and silica gel. You can employ a wide range

Table 59.2 Methods for carbohydrate analysis.

Method	Principle	Comments
Chemical assay		
Benedict's test (and Fehling's test)	Reduction of Cu^{2+} to Cu^+ in presence of reducing sugar; alkaline solution plus heat results in formation of Cu_2O ; solution turns from blue, through yellow, to red	Usually presence/absence test; quantitative assay involves measurement of Cu_2O formed
Dinitrosalicylate (DNS)	Reduction of DNS (yellow) to orange-red derivative; alkaline solution plus heat (100 °C, 10 min)	Quantitative: read at 540 nm
Enzymatic assay		
Glucose oxidase (coupled reaction)	$\beta\text{-D-glucose} + \text{O}_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{H}_2\text{O}_2$ $\text{H}_2\text{O}_2 + \text{reduced dye} \xrightarrow{\text{peroxidase}} \text{H}_2\text{O} + \text{oxidised dye}$	Mutarotation allows reaction to reach completion; hydrogen peroxide formed may be measured using peroxidase; ABTS* is a suitable dye Assay at 437 nm

*ABTS = 2,2'-azino-di-[3-ethylbenzthiazoline]-6-sulfonate.

Measuring carbohydrate migration in TLC systems – the distance migrated by glucose is taken as a reference ($R_F = 100$), and the migration of other carbohydrates is given as the R_G value, where R_G is:

$$\frac{\text{distance moved by carbohydrate}}{\text{distance moved by glucose}} \times 100$$

SAFETY NOTE Working with trimethylsilylating reagents – these compounds are extremely reactive and must be handled with care, using gloves and a fume hood. Since these reagents react violently with water, samples are dried and then redissolved in an organic solvent before trimethylsilylation.

SAFETY NOTE Working with acids – HCl gas is produced when concentrated HCl is heated: always wear gloves and work inside a fume hood.

of traditional solvent systems (Geiss and Morlock, 2022) and your choice of mobile phase will depend upon the expected composition of the mixture, for example, cellulose with ethyl acetate: pyridine : water (100 : 35 : 25 v/v/v) gives a good separation of pentoses and hexoses, and will resolve glucose and galactose, as well as some disaccharides. Staining methods usually exploit the reducing properties of carbohydrates; you can test with reagents such as aniline diphenylamine-phosphoric acid, aniline phthalate and p-anisidine phosphate.

Of the high-resolution techniques, you will find HPLC (p. 349) is the preferred method for the analysis of simple monosaccharide mixtures, and for oligosaccharide analysis and purification. Ion-exchange columns are often used for these purposes, with refractive index, or electrochemical, detection of separated components (Chapter 48). Gas chromatography (GC) is more suitable for complex monosaccharide mixtures, and can analyse subnanomolar amounts of carbohydrates and their derivatives, for example, polyols, including glycerol. However, you will need to add a preliminary step to produce volatile derivatives of the carbohydrates in the mixture. For example, methylation or, more often, trimethylsilylation (adding hexamethyldisilazane and trimethylchlorosilane at 2 : 1 v/v at room temperature rapidly produces trimethylsilyl ethers – TMS derivatives). Efficient separations can be obtained with a non-polar stationary phase, for example, methylpolysiloxo gum (OV-1): the use of SCOT columns (p. 437) can enable over 30 components to be resolved. A disadvantage of GC methods is that the carbohydrates are first converted to a derivative form, then quantified by destructive means, preventing further analysis.

Capillary electrophoresis

Capillary electrophoresis (Volpi, 2010; Chapter 51) is a powerful technique for the separation of carbohydrates, though it is usual to modify uncharged carbohydrates by reductive amination to form primary amines to allow their separation within the capillary.

Examples Complex heteropolymers that contain carbohydrates include:

- **Lignins** – structural polymers in plants: composed of amino acids (phenyl-alanine and tyrosine) together with carbohydrates, and extremely resistant to hydrolysis;
- **Peptidoglycan** – found in the bacterial cell wall (Chapter 38): composed of amino acids and carbohydrates, providing strength and rigidity;
- **Proteoglycans** – found in animal/human connective tissues: composed of a core protein with many carbohydrate side chains attached, responsible for hydration, lubrication, resistance to compressive forces and mediation of cellular interactions.

Characterisation of polysaccharides

You can characterise polysaccharides according to the relative proportions of the constituent sugar residues, the various types of glycosidic links and the M_r . To investigate the composition of a given type of polysaccharide, you must first hydrolyse its glycosidic links (for example, by heating with concentrated HCl at 60 °C for 30 min), followed by separation, identification and quantification of the individual components. You can determine the position of glycosidic linkages by methylation of all free hydroxyl groups of the polysaccharide followed by complete hydrolysis to give a mixture of partially methylated monosaccharides. These are then reduced to alditols, and acetylated. The partially methylated alditol acetates can be identified by GC-mass spectrometry (p. 360), allowing the types of glycosidic links to be deduced from the positions of the acetylated groups. The α and β configuration of the glycosidic linkages can be determined by enzymic assay or by NMR spectroscopy (p. 414).

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STUDY EXERCISES

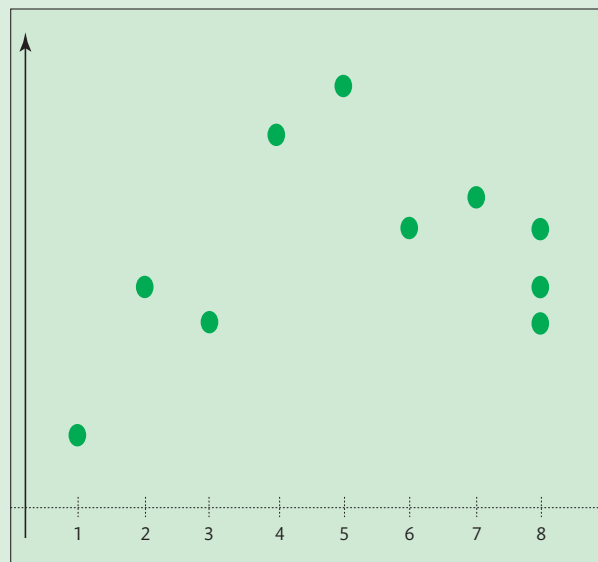
59.1 Check your understanding of the terminology of carbohydrate structure. For each of the following terms: (i) provide a definition; and (ii) give an example of a representative compound:

- aldose;
- pentose;
- hemiacetal;
- pyranoside;
- β anomer.

59.2 Identify sugars by thin-layer chromatography (TLC). A typical TLC separation of sugars on silica gel is shown in the figure opposite.

- Identify the sugars present in the hydrolysed raffinose sample. Check the structure of raffinose, to see whether your findings are consistent with the monosaccharide constituents.
- If the relative frontal mobility of unhydrolysed raffinose relative to glucose (R_G , Chapter 48) is known to be 0.34, and the distance migrated by glucose is 35 mm, how far (in mm) would you expect the raffinose to have migrated?

59.3 Consider the operating principles underlying chemical methods for quantitative assay of carbohydrates. Why is the dinitrosalicylate (DNS) method unsuitable for the assay of the disaccharide sucrose, yet it is suitable for measuring its constituent monosaccharides, i.e. glucose and fructose? Is DNS unsuitable for all disaccharides?



Thin-layer chromatographic separation of sugars: lane 1, lactose; lane 2, glucose; lane 3, galactose; lane 4, xylose; lane 5, ribose; lane 6, fructose; lane 7, arabinose; lane 8, hydrolysed raffinose (dotted line indicates origin and arrow indicates direction of solvent flow).

Answers to these study exercises are available at go.pearson.com/uk/he/resources

60 Assaying nucleic acids and nucleotides

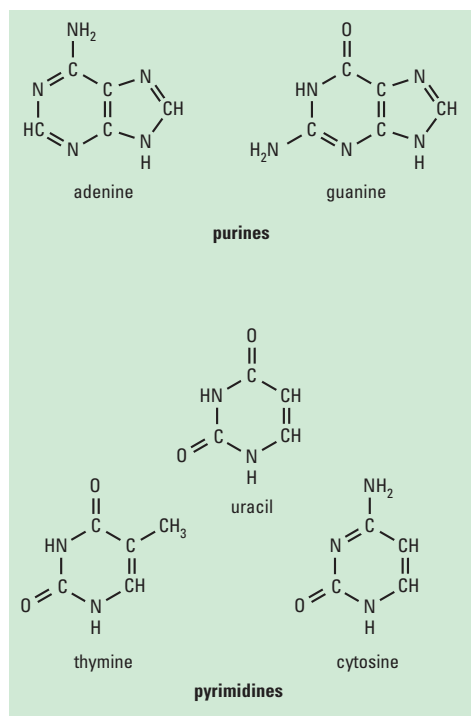


Fig. 60.1 Nitrogenous bases in nucleic acids.

Isolating, characterising and modifying nucleic acids – while the purification and quantification of nucleic acids is covered in this chapter, Chapters 67–69 deal with other aspects of nucleic acid manipulation and analysis, including the isolation of plasmid DNA from bacteria.

Nucleic acids are nitrogen-containing compounds of high M_r , often found within nucleic acid–protein (nucleoprotein) complexes in cells. The two main groups of nucleic acids in eukaryotes are:

1. **deoxyribonucleic acid (DNA)** – found in chromosomes within the nucleus
2. **ribonucleic acid (RNA)** – found in both the cytoplasm and the nucleus.

The chief biological roles of nucleic acids are:

- **Coding** – DNA contains the genetic information of an individual organism (note: in some viruses, this is achieved via RNA – see Chapter 40).
- **Transmission of information** – the different types of RNA are important in the coding and transcription and translation of information within cells, and the creation of proteins from this information.
- **Regulation of gene expression** – some non-coding regions are involved in controlling the transcription and translation of genes.

KEY POINT Nucleic acids are important in the transmission of information within cells, and one of the most important aspects of nucleic acid analysis is to decipher the coded information within these molecules (see also Chapters 67 and 70).

Understanding the structure of nucleic acids

Nucleic acids are polymers of nucleotides (polynucleotides), where each nucleotide consists of:

- **a nitrogenous base**, of which there are five main types. Two have a purine ring structure, i.e. adenine (A) and guanine (G), and three have a pyrimidine ring, i.e. thymine (T), uracil (U) and cytosine (C), as shown in Fig. 60.1. Their carbon atoms are numbered C-1, C-2, etc.
- **a pentose sugar**, which is ribose in RNA and deoxyribose in DNA. The carbon atoms are denoted as C-1', C-2', etc. and deoxyribose has no hydroxyl group on C-2' (Fig. 60.2). The C-1' of the sugar is linked either to the N-9 of a purine or the N-1 of a pyrimidine
- **a phosphate group**, which links with the sugars to form the sugar–phosphate backbone of the polynucleotide chain.

A compound with sugar and base only is called a nucleoside (Fig. 60.2) and the specific names given to the various nucleosides and nucleotides are listed in Table 60.1. The individual nucleotides within nucleic acids are linked by phosphodiester bonds between the 3' and 5' positions of the sugars (Fig. 60.3) giving a polynucleotide chain – the primary structure.

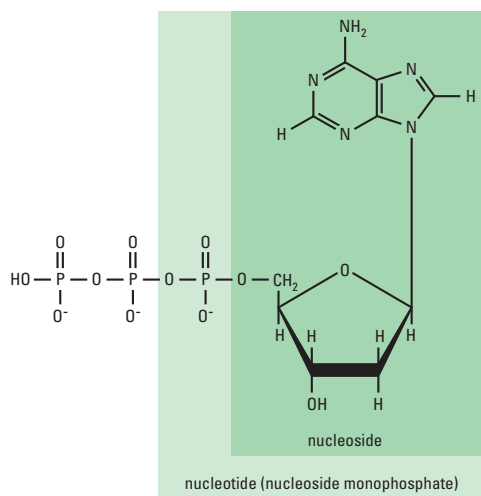


Fig. 60.2 A nucleoside triphosphate – deoxyadenosine 5' triphosphate (dATP).

Table 60.1 Nomenclature of nucleosides and nucleotides.

Base	Nucleoside	Nucleotide
Adenine	Adenosine	Adenylic acid
Guanine	Guanosine	Guanylic acid
Uracil*	Uridine	Uridylic acid
Cytosine	Cytidine	Cytidylic acid
Thymine†	Thymidine	Thymidylic acid

*In RNA.

†In DNA.

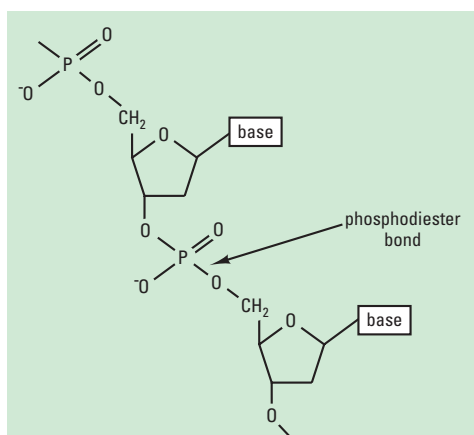


Fig. 60.3 Linkage of nucleotides in nucleic acids.

KEY POINT RNA and DNA differ both in the nature of the pentose sugar residue and in their base composition: both types contain adenine, guanine and cytosine, but RNA contains uracil while DNA contains thymine.

Differences also exist in the conformation of the two types of nucleic acid.

DNA typically exists as two interwoven helical polynucleotide chains (the 'double helix') each running in opposite directions (antiparallel), with respect to the free 3' and 5' ends (Fig. 60.4), with their structure stabilised by hydrogen bonds between matching base pairs on the adjacent strands: A always pairs with T (two hydrogen bonds), and G with C (three hydrogen bonds). This complementarity is important since it stabilises the DNA duplex (double helix) and provides the basis for replication and transcription (Chapter 67). While most double-stranded (ds) DNA molecules are in this form, i.e. as a double helix, some viral DNA is single-stranded (ss). Intact DNA molecules are very large indeed, with high M_r values (for example, 10^9). Further 3D organisation creates tertiary structures, often involved in gene regulation.

Three principal types of RNA exist: messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). mRNA molecules initially synthesised in the nucleus from genomic DNA (nascent mRNA) will contain several sequences – called introns – that are not transcribed into protein. These are successively excised in the nucleus, leaving only coding sequences (exons) in the mRNA that migrates to the cytoplasm, to be translated at the ribosome.

In the main, RNA is single-stranded and in the form of a gentle right-handed helix stabilised by base-stacking interactions, although some sections of RNA (i.e. tRNA) have regions of self-complementarity, leading to base pairing within the single strand and resulting in the formation of hairpin loops of ssRNA between regions of dsRNA (tertiary structure). Each mammalian cell contains about 10 pg of RNA, made up of rRNA (80–85%), tRNA (10–15%) and mRNA (1–5%). While rRNA and tRNA components are of discrete sizes, mRNA is heterogeneous and varies in length from several hundred to several thousand nucleotides. Typical values for M_r of RNA range from 10^4 for tRNA to 10^6 for other types, compared with 10^8 – 10^{11} for DNA.

Extracting and purifying nucleic acids

The types of nucleic acid most commonly isolated are chromosomal DNA, plasmid DNA and mRNA. Irrespective of the source, extraction and purification involve the following stages, in sequence:

- **disruption of cells** to release their contents
- **removal of non-nucleic acid components** (for example, protein), leaving DNA and/or RNA
- **concentration of the remaining nucleic acids.**

DNA isolation procedures

Specific details for the preparation of plasmid DNA from bacterial cells are given in Chapter 69. For other sources of DNA, such as mammalian tissue or plant material, you will need to carry out the following steps:

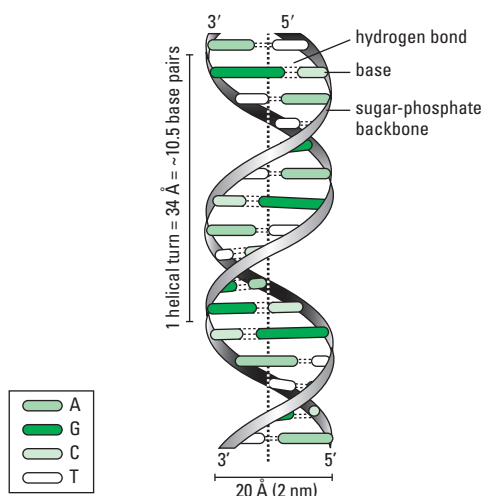


Fig. 60.4 Diagrammatic representation of the double helix arrangement of a portion of DNA. Note that the orientation of the two strands is antiparallel (3' to 5').

Minimising damage to chromosomes – chromosomes vary in size from 0.3 to 200 megabase pairs (Mb), so some breaks in DNA inevitably occur during manipulation. Shear effects can be minimised by using wide-mouthed pipettes, gentle mixing, by avoiding rotamixing and by precipitating DNA with ethanol at -20°C .

Working with precipitated DNA – present in sufficient quantity, this can be spooled from solution by winding it around a glass rod.

Avoiding contamination of glassware by RNases – autoclave all glassware before use, to denature RNases. These enzymes are present in skin secretions: use gloves at all times and use plastic-ware wherever possible.

Avoiding RNase degradation of RNA – endogenous RNases can be inhibited by including diethyl pyrocarbonate (DEP) (at $0.1\% \text{ v/v g mL}^{-1}$) in solutions used for RNA extraction.

- 1. Homogenisation** – tissues can be disrupted by the methods described in detail in Chapter 42, for example, by lysis in a buffered solution containing the detergent sodium dodecyl sulfate (SDS) or Triton X-100.
- 2. Enzymic removal of protein and RNA** – using proteinase (for example, proteinase K at 0.1 mg mL^{-1}) and ribonuclease (typically at $0.1 \text{ } \mu\text{g mL}^{-1}$) for 1–2 h.
- 3. Phenol–chloroform extraction** – to remove any remaining traces of contaminating protein.
- 4. Precipitation of nucleic acids** – usually by adding twice the volume of ethanol.
- 5. Solubilisation** in an appropriate volume of buffer (pH 7.5) – ribonuclease is often added to remove any traces of contaminating RNA.

Density gradient centrifugation (pp. 320–1) is an alternative approach to the separation of DNA from contaminating RNA, as described below.

RNA isolation procedures

Each mammalian cell contains about 10 pg of RNA, made up of rRNA (80–85%), tRNA (10–15%) and mRNA (1–5%). While rRNA and tRNA components are of discrete sizes, mRNA is heterogeneous and varies in length from several hundred to several thousand nucleotides.

KEY POINT RNA is more difficult to purify than DNA, partly because of degradation during the extraction process because of the action of contaminating ribonucleases, and partly because the rigorous treatment required to dissociate the RNA from protein in ribosomes may fragment the polyribonucleotide strands.

You can prepare RNA either from the cytoplasm of cells (to give mainly rRNA, tRNA and fully processed mRNA), or from whole cells, in which case nascent mRNA from the nucleus will also be present.

- To prepare cytoplasmic RNA, you first need to lyse cells or protoplasts with a hypotonic buffer, leaving the nuclei intact. You can then remove any debris arising from the nucleus and rest of the cell by centrifugation (Chapter 44); sodium dodecyl sulfate (SDS) should be added to the supernatant (the cytoplasmic fraction) to inhibit ribonuclease. Proteinase K can be added to release rRNA from ribosomes. You can carry out phenol–chloroform extraction to remove contaminating proteins, as for DNA preparation, and the RNA present in the aqueous phase can then be precipitated by addition of twice the volume of ethanol.
- To prepare whole-cell RNA you will need to carry out more vigorous cell lysis, for example, with a solution containing 6 mol L^{-1} guanidinium chloride and 2-mercaptoethanol: this effectively denatures any ribonuclease present. Caesium chloride is added to the extract to give a final concentration of 2.4 mol L^{-1} , and the solution is processed by density gradient centrifugation (Chapter 44) at $100\,000 \text{ g}$ for 18 h,

Purification of mRNA – following whole cell extraction of RNA, mRNA can be separated from other types by affinity chromatography (p. 355) using poly (U)-Sephrose which binds to the poly (A) 'tail' sequence at the 3' end of mRNA molecules.

using a cushion of 5.7 mol L^{-1} CsCl. DNA and protein remain in the upper layers of CsCl, while RNA forms a pellet at the base of the tube. The RNA pellet is redissolved in buffer, and precipitated in cold ethanol.

Separating nucleic acids

Electrophoresis is the principal method used for separating nucleic acids. At alkaline pH values, linear DNA and RNA molecules have a uniform net negative charge per unit length owing to the charge on the phosphoryl group of the backbone. Electrophoresis using a supporting medium that acts as a molecular sieve (for instance, agarose or polyacrylamide, pp. 367–8) enables DNA fragments or RNA molecules to be separated on the basis of their relative sizes (see Chapter 50 for further details).

Quantitative analysis of nucleic acids

Measuring nucleic acid content

You can measure the concentration of reasonably pure samples of DNA or RNA relatively easily by spectrophotometry (p. 448), using a standard of known concentration to calibrate the assay. In contrast, you will find that measurement of the nucleic acid content of whole cell or tissue homogenates is best achieved using chemical methods, since the homogenates will contain many interfering substances. The principles involved in each technique are as follows:

Measuring nucleic acids by spectrophotometry – ideally, nucleic acid extracts should be prepared to give A_{260} values of between 0.10 and 0.50, for maximum accuracy and precision.

SAFETY NOTE Working with ethidium bromide – this compound is highly toxic and mutagenic. Avoid skin contact (wear gloves) and avoid ingestion. Use a safe method of disposal (e.g. adsorb from solution using an appropriate adsorbant, e.g. activated charcoal). Less toxic alternatives include SYBR Safe and Gel Red.

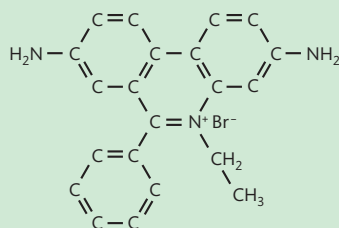


Fig. 60.5 Ethidium bromide (EtBr), a fluorescent molecule used for the detection and assay of DNA.

- **Spectrophotometry** – DNA and RNA both show absorption maxima at $\approx 260 \text{ nm}$, owing to the conjugated double bonds present in their constituent bases. At 260 nm , an A_{260} value of 1.0 is given by a $50 \mu\text{g mL}^{-1}$ solution of dsDNA, or a $40 \mu\text{g mL}^{-1}$ solution of ssRNA. If you also measure the absorbance at 280 nm , protein contamination can be quantified. Pure nucleic acids give A_{260}/A_{280} ratios of 1.8–2.0, and a value below 1.6 indicates significant protein contamination. You will need to carry out further purification steps for contaminated samples, for example, by repeating the phenol–chloroform extraction step. RNA contamination of a DNA preparation is indicated if A_{260} decreases when the sample is treated with $2.5 \mu\text{L}$ of RNase at $20 \mu\text{g mL}^{-1}$. DNA contamination of an RNA preparation might be suspected if the sample is very viscous, and this can be confirmed by electrophoresis.

- **Spectrofluorimetry** – you will find that this is the best approach for samples where the DNA concentration is too low to allow direct assay by the spectrophotometric method described above. The traditional method uses the fluorescent dye ethidium bromide (Fig. 60.5), which binds to dsDNA by insertion between stacked base pairs, a phenomenon termed 'intercalation'. The fluorescence of ethidium bromide is enhanced 25-fold when it interacts with dsDNA. Since ssDNA gives no significant enhancement of fluorescence, dsDNA can be quantified in the presence of denatured DNA. You can calculate the concentration of dsDNA in solution, $[\text{dsDNA}]_x$, by comparing its fluorescence (excitation, 525 nm ; emission, 600 nm) with that of a standard of known concentration, $[\text{dsDNA}]_{\text{std}}$, using the relationship:

$$[\text{dsDNA}]_x = \frac{[\text{dsDNA}]_{\text{std}} \times \text{fluorescence of unknown}}{\text{fluorescence of standard}} \quad [60.1]$$

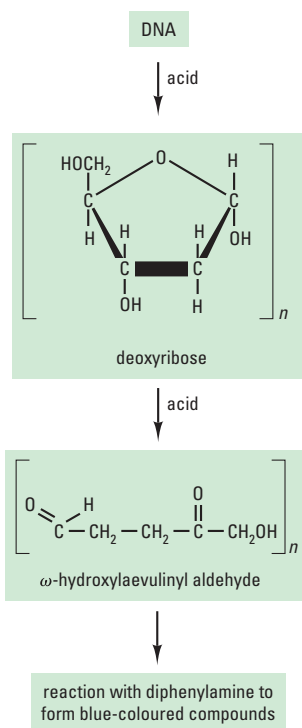


Fig. 60.6 The diphenylamine reaction for assay of DNA.

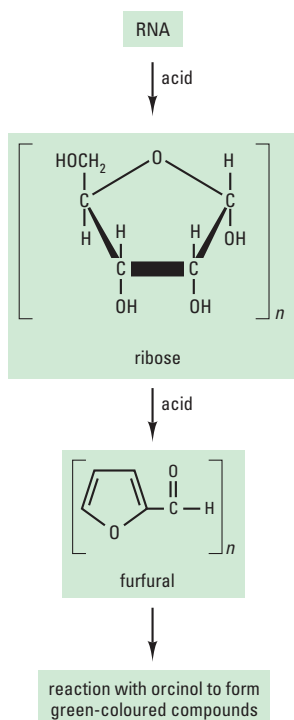


Fig. 60.7 The orcinol reaction for assay of RNA.

- Chemical methods** – these are mostly based on colorimetric reactions with the pentose groups of nucleic acids. You can measure the total DNA concentration by the diphenylamine reaction (Fig. 60.5), which is specific for 2-deoxypentoses. This involves heating 2 mL of DNA solution with 4 mL of freshly prepared diphenylamine reagent (diphenylamine, at 10 gL^{-1} in glacial acetic acid, plus 25 mL concentrated sulfuric acid) for 10 min in a boiling water bath. The acids cleave some of the phosphodiester bonds, and hydrolyse the glycosidic links between the deoxyribose and purines. Deoxyribose residues are converted to ω -hydroxyaevalvinyl aldehyde (Fig. 60.6), which reacts with the diphenylamine to produce a blue pigment, assayed at 600 nm. By constructing a DNA standard curve ranging from 0 to $400 \mu\text{g mL}^{-1}$, the DNA concentration of the unknown can be determined.

You can measure the RNA concentration by the orcinol reaction, which is a general assay for pentoses. This involves heating 2 mL of RNA solution with 3 mL of orcinol reagent (prepared by dissolving 1 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1 litre of concentrated HCl, and adding 35 mL of 6% w/v orcinol in ethanol) in a boiling water bath for 20 min. The acid cleaves some phosphodiester bonds and hydrolyses the glycosidic links between the ribose and purines. The hot acid also converts the ribose to furfural (Fig. 60.7), which reacts with orcinol in the presence of ferric ions to produce green-coloured compounds, assayed at 660 nm. A standard curve for RNA ranging from 0 to $400 \mu\text{g mL}^{-1}$ is used to determine the RNA concentration of the unknown. The orcinol reaction is less specific than the diphenylamine reaction, as deoxyribose reacts to some extent and DNA gives about 10% of the colour given by the same concentration of RNA. If you know the DNA concentration of the extract (for instance, from a diphenylamine assay), the contribution of DNA to A_{660} can be measured for a standard prepared to have the same DNA concentration as the sample: the A_{660} due to DNA can then be subtracted from the result of the orcinol reaction, and the remaining A_{660} value is then used to determine RNA concentration from the RNA standard curve.

Assaying the relative proportions of base pairs in DNA

Double-stranded DNA has a lower molar absorptivity at 260 nm than single-stranded DNA, owing to electron interactions between the stacked base pairs and hydrogen bonding between the complementary bases of dsDNA. When you heat a dsDNA solution slowly, there is little change in absorbance until a temperature is reached where the hydrogen bonds are broken and the DNA strands separate. At this so-called 'melting temperature' (T_m), the A_{260} value increases sharply (Fig. 60.8). This temperature-dependent increase in absorbance is often referred to as the 'hyperchromic effect'. The actual value of the T_m (and hence the stability of dsDNA) is dependent on the base pair composition of the DNA being studied: GC base pairs have three double bonds while AT base pairs have two, and the higher the % GC content, the higher the value of the T_m . The length of the molecule also affects the T_m , since short dsDNA molecules do not show a hyperchromic effect. Studies on a number of DNA samples have shown the following relationship, for GC contents between 30 and 70%:

$$\% \text{ GC} = 2.44(T_m - 69.3) \quad [60.2]$$

The relative proportion of AT base pairs can then be calculated by subtracting the % GC value from 100, giving % AT.

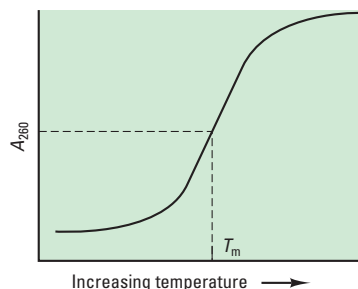


Fig. 60.8 Effect of temperature on the absorbance (A_{260}) of DNA in solution. T_m is the temperature for the mid-point of the absorbance change.

Sources for further study

(See also Chapter 70 for nucleic acid databases and related information.)

Blackburn, G.M., Gait, M.G., Loakes, D. and Williams, D.M. (2005) *Nucleic Acids in Chemistry and Biology*, 3rd edn. Royal Society of Chemistry, London.

Dhaliwal, A. (2020) *DNA Extraction and Purification*. Available: [//dx.doi.org/10.13070/mm.en.3.191](https://doi.org/10.13070/mm.en.3.191). Last accessed 13/05/21.

Kerr, J.T. and Birch, L. (eds) (2008) *Essentials of Nucleic Acid Analysis: A Robust Approach*. Royal Society of Chemistry, London.

Neidle, S. (2007) *Principles of Nucleic Acid Structure*, 2nd edn. Academic Press, Oxford.

Rapley, R. (2000) *The Nucleic Acids Protocols Handbook*. Humana Press, New Jersey.

STUDY EXERCISES

60.1 Calculate the concentration of DNA in a sample using a fluorescence assay (see also exercises 47.2c and 47.2d, for equivalent exercises using UV spectrophotometry). A volume of 10 μL of a DNA standard solution (of concentration 50 $\mu\text{g mL}^{-1}$) was added to 3 mL of an ethidium bromide solution (containing 0.5 mgL^{-1} ethidium bromide, buffered at pH 7.5) in a fluorimeter cuvette, mixed, then read in a spectrofluorimeter using an excitation wavelength of 525 nm and an emission wavelength of 600 nm, giving a relative fluorescence intensity (RFI) of 58. This was repeated using 10 μL of a DNA solution of unknown concentration instead of the DNA standard solution, giving an RFI of 32. What is the DNA concentration of the unknown? (Express your answer to three significant figures.)

60.2 Investigate the binding properties of ethidium bromide. Find out: (i) why ethidium bromide fluorescence is enhanced by DNA; and (ii) under what circumstances ethidium bromide can also bind to RNA.

60.3 Consider how to avoid problems in DNA spectrofluorometric analysis caused by the presence of RNA in a sample. What steps could you take to ensure that all the fluorescence obtained from a cell extract was caused by the reaction of ethidium bromide with DNA rather than from DNA plus contaminating RNA?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

61 Protein purification

Much of our current knowledge about metabolic and physiological events has been gained from *in vitro* studies of purified proteins. Such studies range from investigations into the kinetics and regulation of enzymes (Chapter 62) to the determination of the structure of a protein and its relationship to function. In addition, certain purified proteins have a role as therapeutic agents; for example, Factor VIII in the treatment of haemophilia.

Applying protein purification techniques in proteomics – an important aspect of proteomics (p. 536) is the characterisation of individual proteins, using some of the techniques described in this chapter.

KEY POINT The purification of most proteins involves a series of procedures, based on differential solubility and/or chromatography, that selectively separate the protein of interest from other proteins and materials. It is rare for purification to be a one-step process.

Deciding on your objectives

Purity, yield and cost are the major considerations, and the relative importance of these factors will depend on the purpose of the purification. For studies on the biological activity of a protein (for example, an enzyme), only microgram amounts may be required and, as long as there are no interfering substances present, 100% purity may not be necessary. For structural studies, milligram amounts may be needed and the protein must be pure. A protein produced for industrial applications (for example, an enzyme for use in starch degradation) needs to be produced in large amounts (grams or kilograms), but purity is not usually essential. In contrast, commercially produced proteins for therapeutic use need to be free of any significant contamination.

The cost of the purification will depend on the nature of the source material, the number of purification steps required and the price of materials for the separation techniques employed. Inevitably, some protein will be lost at each stage of the purification procedure.

KEY POINT You should aim to use the smallest number of purification steps that will give you the yield and purity required for your application.

Preliminary considerations

Investigating what is known about the protein

Rather than approaching every purification step on a ‘trial and error’ basis, try to find out as much as you can about the physical and biological properties of the desired protein before you begin your practical work. Even if the protein to be purified is novel, it is likely that similar proteins will be described in the literature. Information on the isoelectric point (pI) and M_r of both the protein and of the likely major contaminants, is particularly useful for ion-exchange and gel permeation chromatography (p. 453). Knowledge of other factors such as metal ion and co-enzyme requirements, presence of thiol groups and known inhibitors can indicate useful chromatographic steps, and may allow you to take steps to preserve

Definition

Isoelectric point (pI) – the pH value at which a protein has a net charge of zero, i.e. where positive and negative charges on amino acid side chains are balanced.

Extracting protein fractions from tissues and cells – relevant approaches for selecting source material and initial separation techniques are discussed in Chapter 57.

Storing protein solutions during a purification procedure – overnight storage at 4 °C is acceptable, but it is advisable to include bacteriostatic agents (e.g. azide at 0.5% w/v) and protease inhibitors: freezing may be required for longer term storage – use liquid nitrogen or dry ice/methanol for rapid freezing. If freezing and thawing might denature the sample, include 20% v/v glycerol in buffers, to allow storage at –20 °C in liquid form.

Working with recombinant proteins – these are often produced with either N-terminal or C-terminal hexahistidine 'tags', enabling them to be purified readily using immobilised metal affinity chromatography (p. 355).

Measuring yield – note that yields of >100% may be obtained if an inhibitor is lost during purification.

Table 61.1 Principal causes of decreased yield in protein purification.

Cause	Possible solution
Denaturation	Include EDTA or reducing agents in buffers; avoid extreme temperatures
Inhibition	Check buffer composition for possible inhibitors
Proteolysis	Include protease inhibitors in buffers
Non-elution	Alter salt concentration or pH of the eluting buffer
Co-factor loss	Recombine fractions on a trial-and-error basis

the tertiary structure and biological activity of the desired protein during the purification process.

Devising a strategy for protein purification

Any successful protein purification scheme will exploit the unique properties of the desired protein in terms of its size, net charge, hydrophobic nature, biological activity, etc. The chromatographic techniques that separate on the basis of these properties are detailed in Chapters 48–50. However, the *order* in which the various purification steps are carried out needs some thought, and each stage needs to be considered in relation to the following factors:

- **Capacity** – the amount of material (volume or concentration) that the technique can handle. High-capacity techniques such as ammonium sulfate precipitation (see later) and ion-exchange chromatography (p. 351) should be used at an early stage, to reduce the sample volume.
- **Resolution** – the efficiency of separating one component from another. At later stages of the purification, the sample volume will be considerably reduced, but any contaminating proteins may well have very similar properties to those of the protein of interest. Therefore, a high-resolution (but low capacity) technique will be required, such as covalent chromatography or immobilised metal affinity chromatography (IMAC).
- **Yield** – the amount of protein recovered at each step, expressed as a percentage of the initial amount (p. 453). For example, yields of >80% can be obtained with ammonium sulfate precipitation. With certain types of affinity chromatography, where harsh elution conditions need to be employed, yields may be quite low (for example, <20%). Some of the other possible reasons for a decreased yield are considered in Table 61.1.

An ideal purification procedure will have the following features:

1. **an initial step that has a high capacity**, but not necessarily a high resolution
2. **a series of chromatographic steps**, each of which exploits a different property of the protein to increase purity
3. **a minimum number of manipulations** and changes in conditions (for example, in buffer composition) between steps.

The following series of steps would meet the above conditions:

- Step 1 Ammonium sulfate precipitation** – a high-capacity, low-resolution technique that yields a protein solution with a greatly reduced volume and a high concentration of ammonium sulfate.
- Step 2 Hydrophobic interaction chromatography (HIC)** – an absorption technique (pp. 352–3) using a starting buffer with a high ammonium sulfate concentration. During development of the column, the ionic strength of the mobile phase is reduced in a gradient elution.
- Step 3 Ion-exchange chromatography (IEC)** – another absorption technique (pp. 351–2). Here the starting buffer has a low ionic strength, and a gradient of increasing ionic strength is used to separate components.

Calculating total protein content –

multiply the protein concentration by the total volume, making sure that the units are consistent, e.g. $\text{mg mL}^{-1} \times \text{mL}$ to give total protein content in mg. A similar procedure is required to determine the total amount of enzyme.

Using immunoassays – note that inactive or denatured forms of the protein may be detected along with the biologically active form.

Example For an enzyme extract having a specific activity of 250.2 U mg^{-1} compared to an initial specific activity of 45.5 U mg^{-1} , using eqn [61.1] gives a purification factor of $250.2 \div 45.5 = 5.5$ -fold (to one decimal place).

Example For an enzyme extract having a total activity of 8521 U compared to an initial total activity of 9580 U, using eqn [61.2] gives a yield of $[8521 \div 9580] \times 100 = 88.9\%$ (to one decimal place).

Step 4 Gel permeation chromatography (GPC) – a low-capacity method (p. 352) where separation is independent of the composition of the mobile phase.

The sample from step 1 may be able to be applied directly to the HIC column, and that from step 2 may be used for IEC without changing the buffer. A concentration step (pp. 454–5) would be required before step 4. The above scheme is very much an idealised approach: in practice, buffers may need to be changed by dialysis or ultrafiltration, as described later. However, the principle of using the minimum number of manipulations to obtain the desired purification remains valid.

Monitoring purification

At each step, the separated material is usually collected as a series of ‘fractions’. Each fraction must be assayed for the protein of interest, and fractions containing that protein are pooled prior to the next step. The assay performed will depend on the properties of the protein of interest, but specific enzyme assays or immunoassays are most commonly used. The protein concentration (pp. 426–7) and the volume of the pooled fraction must be determined, and these values, together with those obtained for the amount of the protein of interest, are used to determine the purity and yield after each step. For enzymes, the biological activity is measured and used to calculate the specific activity (the enzyme activity per unit mass of protein) as described on p. 459. By determining the specific activity of the enzyme at each step, the degree of purification, or purification factor (*n*-fold purification), can be obtained from this relationship:

$$\text{Purification factor} = \frac{\text{specific activity after a particular step}}{\text{specific activity of initial sample}} \quad [61.1]$$

Increased purification usually represents a decrease in total protein relative to the biological activity of the protein of interest, though in some instances it may reflect the loss of an inhibitor during a purification step.

Calculation of the yield of enzyme at each step is straightforward, since:

$$\text{Yield} = \frac{\text{total enzyme activity after a particular step}}{\text{total enzyme activity in initial sample}} \times 100 (\%) \quad [61.2]$$

Note that the yield equation uses the *total amount* of enzyme and is therefore unaffected by the volume of the solutions involved. You should make a record of the progress of your purification procedure at each step, as in Table 61.2.

Monitoring progress using electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of subsamples from each step will give an indication of the number of proteins still present: ideally, a pure protein will give only one band after silver staining (pp. 371–2); however, this stain is so sensitive that even trace impurities can be detected in what you expect to be a pure sample, so do not be dismayed by the appearance of the gel after staining.

Carrying out SDS-PAGE and isoelectric focusing (or, ideally, 2D-PAGE, p. 376) on subsamples also gives information on the M_r and pI of any

Table 61.2 Example of a record of purification for an enzyme.

Step	Procedure	Total protein (mg)	Total enzyme (U)*	Specific activity (U mg ⁻¹)	Purification factor (n-fold)	Enzyme yield (%)
Initial sample	–	210	1984	9.45	–	100.0
Step 1	'Salting out'	112	1740	15.54	1.6	87.7
Step 2	HIC	30	1701	56.70	6.0	85.7
Step 3	IEC	6.0	1604	267.33	28.3	80.8
Step 4	GPC	3.0	1550	516.66	54.7	78.1

*U = unit (p. 459). HIC = hydrophobic interaction chromatography (pp. 352–3); IEC = ion-exchange chromatography (pp. 351–2); GPC = gel permeation chromatography (p. 352).

Assaying enzymes – if possible, use a preliminary screening technique to detect active fractions prior to quantitative assay, e.g. using tetrazolium dyes in microtitre plates to detect oxidoreductases (p. 373).

Measuring the mass of proteins – this is sometimes expressed in daltons (Da), or kilodaltons (kDa), where 1 dalton = 1 atomic mass unit. M_r is an alternative, numerically equivalent, expression (p. 153).

Using ammonium sulfate – although the phenomenon of salting out is seen with several salts, $(\text{NH}_4)_2\text{SO}_4$ is the most widely used because it is highly soluble (saturating at $\approx 4 \text{ mol L}^{-1}$), inexpensive and can be obtained in very pure form.

remaining contaminants, and this can be taken into account when planning the next chromatographic step.

Differential solubility separation techniques

Often the volume and protein concentration of the initial soluble extract will be quite high. Application of a differential solubility technique at this stage results in the precipitation of selected proteins. These can be recovered by filtration or centrifugation, washed and then resuspended in an appropriate buffer. This will reduce the sample volume and may give a small degree of purification, making the sample more suitable for subsequent chromatographic steps.

Ammonium sulfate precipitation ('salting out')

This is the most widely used differential solubility technique, having the advantage that most precipitated enzymes are not permanently denatured, and can be redissolved with restoration of activity. Precipitation depends on the existence of hydrophobic 'patches' on the surface of proteins, inducing a reorganisation of water molecules in their vicinity. When ammonium sulfate is added to the extract, it dissolves to give ions that become hydrated, leaving fewer water molecules in association with the protein. As a result, the hydrophobic patches become 'exposed', and hydrophobic interactions between different protein molecules lead to their aggregation and precipitation. The basis of fractionation in this method is that, as the salt concentration of the extract is increased, proteins with larger or more abundant hydrophobic patches will precipitate before those with smaller or fewer patches.

Unless you can obtain information from the literature about the $(\text{NH}_4)_2\text{SO}_4$ concentration that will precipitate your target protein, fractionation is done on a trial-and-error basis. The $(\text{NH}_4)_2\text{SO}_4$ concentration is expressed in terms of the percentage saturation value: Table 61.3 shows the amount of $(\text{NH}_4)_2\text{SO}_4$ required to give 20–100% saturation. For each percentage saturation chosen, the $(\text{NH}_4)_2\text{SO}_4$ salt should be added slowly while stirring, and the mixture left at 4 °C for 1 h before centrifuging at 3000 g for 40 min. For an effective separation, start with the maximum percentage saturation that does not precipitate the protein of interest, then increase the percentage saturation by the minimum amount that will then precipitate it. The proteins precipitated between any two values of percentage saturation (say between 30 and 50%) are referred to as a 'cut'. An alternative approach is to add saturated $(\text{NH}_4)_2\text{SO}_4$ solution – the volume (V_a) to be added to an

Example To prepare a solution of 40% $(\text{NH}_4)_2\text{SO}_4$ saturation ($S_f = 0.4$) from 100 mL (V_i) containing no added $(\text{NH}_4)_2\text{SO}_4$ using eqn [61.3]:
 $V_a = 100(0.4 - 0) \div (1 - 0.4) = 67$ mL of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, giving a volume of 167 mL.
 For a 40–60% 'cut' of this sample ($V_i = 167$ mL), using eqn [61.3]:
 $V_a = 167(0.6 - 0.4) \div (1 - 0.6) = 83.5$ mL of saturated $(\text{NH}_4)_2\text{SO}_4$ solution, to give a final volume of 250.5 mL.

Purifying bacterial proteins – adjusting the extract to pH5 can be useful, since many bacterial proteins have pI values in this region.

Example The HPII catalase of stationary phase *E. coli* remains intact when heated to 55 °C for 15 min, aiding its separation from other proteins.

Table 61.3 Amount of $(\text{NH}_4)_2\text{SO}_4$ (g L^{-1}) required for a particular percentage saturation.

Final concentration (%) →	20	30	40	50	60	70	80	90	100
Initial concentration (%) ↓	Ammonium sulfate added (g L^{-1})								
0	107	166	229	295	366	442	523	611	707
10	54	111	171	236	305	379	458	545	636
20	–	56	115	177	244	316	392	476	565
30		–	57	119	184	253	328	408	495
40			–	59	122	190	262	340	424
50				–	61	127	197	272	353
60					–	63	131	204	283
70						–	66	136	212
80							–	68	141
90								–	71

initial volume of solution (V_i) with an initial saturation S_i , to give a final saturation S_f , is given by the equation:

$$V_a = \frac{V_i(S_f - S_i)}{1 - S_f} \quad [61.3]$$

where S_i and S_f are expressed as fractional saturation, for example, $S_f = 0.5 = 50\%$ saturation, and both volumes are expressed in the same terms, for example millilitres.

Precipitation by changing pH

Proteins are least soluble at their isoelectric points because, at that pH, there is no longer the repulsion that occurs between positively or negatively charged protein molecules at physiological pH values. If the precipitated proteins are required for further purification, it is essential that the protein of interest is not irreversibly denatured. The method is probably best employed by precipitating contaminating proteins, leaving the desired protein in solution. Use citric acid for <pH 3, acetic acid for <pH 4 and sodium carbonate or ethanolamine for >pH 8.

Heat denaturation

Exposure of most proteins to high temperatures disrupts their conformation through effects on non-covalent interactions such as hydrogen bonds and van der Waals forces. However, different proteins are denatured, and hence precipitated, at different temperatures, and this can provide a basis for the separation of some heat-stable proteins. By incubating small aliquots (≈ 1 mL) of extract for 1 min at a range of temperatures between 45 and 65 °C, it is possible to determine the temperature that gives maximum precipitation of contaminating protein with minimal inactivation of the desired protein.

Solvent and polymer precipitation methods

Organic solvents (for example, acetone, ethanol) cause precipitation of proteins by lowering the dielectric constant of the solution. Performing

Definition

Dielectric constant (ϵ) – a dimensionless measure of the screening effect on the force (F) between two charges (q_1 , and q_2) owing to the presence of solvent, from the equation $F = (kq_1q_2) \div (\epsilon r^2)$, where r is the distance between the two particles and k is a constant. The dielectric constant for water is high (≈ 80), while most organic solvents have lower values, in the range 1–10.

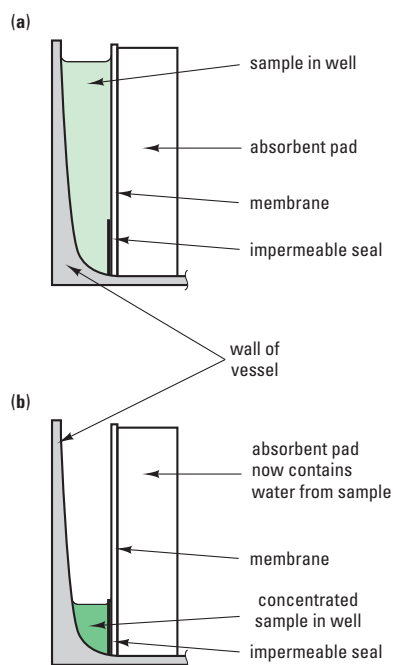


Fig. 61.1 Minicon ultrafiltration system (a) with sample added, (b) after ultrafiltration.

Using Visking tubing – this must be boiled before use to ensure a uniform pore size and to remove heavy metal contaminants.

Avoiding protein precipitation during dialysis or gel filtration – make sure your buffer pH is either above or below the pI of the proteins.

the precipitation at 0 °C minimises permanent denaturation. Step-wise concentration (% v/v) increments are used, giving ‘cuts’ of precipitated proteins, as with ammonium sulfate precipitation.

Organic polymers, particularly polyethylene glycol (PEG), also lower the dielectric constant, but at lower concentrations than with acetone or ethanol. The most commonly used PEG preparations have M_r values of 6000 or 20 000, and these can be removed from the sample by ultrafiltration. PEG precipitation does not involve salts, so it may be a useful preliminary step prior to ion-exchange chromatography, which starts with low salt buffer. Also, since the techniques of PEG and ammonium sulfate precipitation involve different principles, they can be used sequentially.

Concentration by ultrafiltration

This involves forcing water and small molecules through a semi-permeable membrane using high pressure or centrifugation. A range of membranes with ‘nominal’ molecular weight cut-offs between 500 and 300 000 are commercially available (for example, Amicon, Millipore), with pore sizes of 0.1–10 μm . Concentration of small samples (<5 mL) can be achieved using either a membrane backed by an absorbent pad (for example, Minicon, Fig. 61.1, available with M_r cut-off from 5000 to 30 000), or by using a centrifugal concentrator (for example, Vivaspin, Centricon). Larger volumes (up to 400 mL) can be concentrated using a stirred ultrafiltration chamber (an ultrafiltration ‘cell’) where the liquid is forced through the membrane using nitrogen or an inert gas.

Ultrafiltration not only concentrates the sample, but also may give a degree of purification. It can also be used to change the buffer composition by diafiltration (p. 456). Note that molecular weight cut-off values are quoted for globular proteins – fibrous proteins of higher M_r may pass through the ultrafiltration membrane.

Removing salts and changing the buffer

Although you should aim to use the minimum number of manipulations to obtain the desired purification, it may be necessary to remove salts or to change the buffer before the next step will work effectively (for example, when carrying out IEC, the ionic strength or the pH of the sample may need to be changed before the target protein will bind to the column). Several methods are available, including the following:

- **Dialysis:** the sample is placed in a bag consisting of semi-permeable membrane (for example, Visking tubing) and placed in at least 20 volumes of the required buffer. The membrane allows molecules of $M_r < 20\,000$ to pass freely, while retaining larger molecules (note that dialysis is not suitable for small proteins). The small molecules diffuse through the membrane until the osmotic pressure between the sample and the dialysis buffer is equalised. Several changes of dialysis buffer may be required to obtain the desired buffer conditions within the sample. Normally dialysis is carried out overnight at 4 °C. Efficient stirring is required – use a magnetic stirrer and stirrer bar.
- **Diafiltration:** quicker than dialysis and more suitable for larger volumes. It involves addition of the desired buffer to the sample solution, followed by ultrafiltration. Several buffer addition and ultrafiltration steps may be necessary to obtain the desired sample conditions.

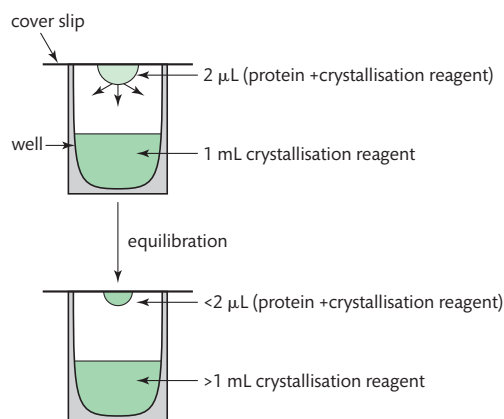


Fig. 61.2 The hanging drop technique for protein crystallisation.

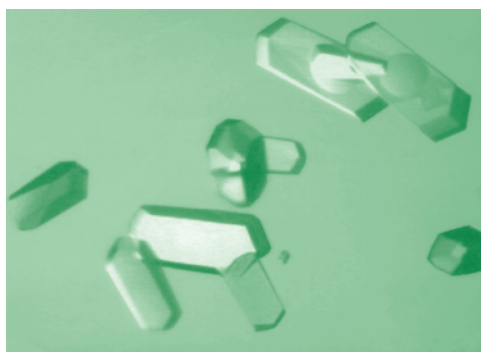


Fig. 61.3 Image of protein crystals produced by the hanging drop method.

Image courtesy of S. Charnock.

- **Gel permeation chromatography** (gel filtration): a gel filtration medium of small pore size (for example, Sephadex G-25) is used to prepare a column of ≈ 5 times the volume of the sample. When the sample passes through the column, the large protein molecules will elute with the void volume, while salt ions are retained. This method is only suitable for small volumes, and it results in dilution of the sample.

Protein crystallisation

Much of the information on protein structure that is now available on protein sequence databases (see Table 70.1) has been obtained by X-ray crystallographic studies on protein crystals. To obtain protein crystals, the protein should be purified to at least 90–95% and its concentration in solution should be $10\text{--}20\text{ mg mL}^{-1}$. The method most widely used for crystallisation is the hanging drop vapour diffusion technique (Fig. 61.2). Here, a small droplet of the protein sample (usually about $1\text{--}2\text{ }\mu\text{L}$) is mixed with a crystallisation reagent on a siliconised glass coverslip and inverted over a microtitre plate well containing $0.5\text{--}1.0\text{ mL}$ of the same crystallisation reagent. The presence of protein in the droplet means that the initial concentration of crystallisation reagent in the droplet is less than that in the well. As a result, water will diffuse from the droplet into the vapour phase until an equilibrium is achieved between the droplet, the vapour phase and the solution in the well. During this equilibration, the protein is concentrated within the droplet, enhancing the formation of protein crystals. The essential components of a crystallisation reagent are: (i) protein precipitant, and (ii) buffer. The specific reagents differ, depending on the type of protein to be crystallised. Commercially available reagents include the PEG/Ion Screen reagents (Hampton Research, USA) and the Clear Strategy Screen reagents (Molecular Dimensions, UK). An image of protein crystals produced by this method is shown in Fig. 61.3.

Sources for further study

Ahmed, H. (2016) *Principles and Reactions of Protein Extraction, Purification and Characterisation*, 2nd edn. CRC Press, Boca Raton.

Coligan, J.E., Dunn, B.M., Speicher, D.W. and Wingfield, P.T. (2003) *Short Protocols in Protein Science*. Wiley, New York.
[Provides details of over 500 specific methods.]

Hunte, C, von Jagow, G. and Schagger, H. (2003) *Membrane Protein Purification and Crystallisation: a Practical Guide*, 2nd edn. Academic Press, San Diego.
[Gives specific procedures for isolation of membrane proteins.]

Rosenberg, I.M. (2005) *Protein Analysis and Purification: Benchtop Techniques*, 2nd edn. Springer-Verlag, Berlin.

Scopes, R.K. (2010) *Protein Purification: Principles and Practice*, 3rd edn. Springer, New York.

Simpson, R.J., Adams, P.D. and Golemis, E.A. (2008) *Basic Methods in Protein Purification and Analysis: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Wilkins, M.R., Appel, R.D., Williams, K.L. and Hochstrasser, D.F. (eds) (2008) *Proteome Research: Concepts Technology and Application (Principles and Practice)*, 2nd edn. Springer, New York.

STUDY EXERCISES

61.1 Practise ammonium sulfate fractionation calculations. For a mixture of three proteins, A, B and C, in 100 mL of aqueous solution, you are provided with the information that protein A precipitates at an $(\text{NH}_4)_2\text{SO}_4$ concentration of 40%, and that protein B precipitates in the 40–60% 'cut', while protein C is not precipitated by 60% $(\text{NH}_4)_2\text{SO}_4$. Determine how much solid $(\text{NH}_4)_2\text{SO}_4$ (in grams) would be needed to achieve 40% saturation and, once protein A is removed, how much $(\text{NH}_4)_2\text{SO}_4$ will then need to be added to the solution to reach 60% saturation. What will be the fate of protein C in this procedure?

61.2 Select a chromatographic technique for use with a particular protein. Using the information given in Chapter 48, which affinity techniques would be most suitable for the following proteins: (a) an enzyme that uses NAD^+/NADH as a co-factor, (b) a genetically engineered protein with a polyhistidine tag at its N-terminal end, (c) a glycoprotein?

61.3 Calculate yield and relative purification of an enzyme. The table shows representative data for an enzyme purification involving five stages. Calculate the yield and n -fold purification (relative to the initial values) at each stage. Give all answers to three significant figures.

Volume, protein concentration and enzyme activity at several stages during a protein purification procedure.

Stage	Total volume of extract (mL)	Enzyme activity (U mL^{-1})	Protein concentration (mg mL^{-1})
1	78	1.2	4.8
2	70	1.2	1.7
3	10	6.4	2.5
4	5	10.8	1.4
5	0.6	65.7	3.4

Answers to these study exercises are available at go.pearson.com/uk/he/resources

62 Studying enzymes

Enzymes are globular proteins that increase the rate of specific biochemical reactions. Each enzyme operates on a limited number of substrates of similar structure to generate products under well-defined conditions of concentration, pH, temperature, etc. In metabolism, groups of enzymes work together in sequential pathways to catalyse complex molecular transformations, for example, the multi-reaction conversion of glucose to lactate in glycolysis.

Example Enzyme EC 1.1.1.1 is usually known by its trivial name, alcohol dehydrogenase.

KEY POINT Enzymes are categorised according to the chemical reactions they catalyse, leading to a four-figure Enzyme Commission (EC) code number and a systematic name for each enzyme. Most enzymes also have a recommended trivial name, often denoted by the suffix 'ase'.

Measuring enzyme reactions – important parameters

Activity

Example An enzyme preparation that converted 295 μmol of substrate to product in 15 min would have an activity, expressed in terms of non-SI units (U) of: $295 \div 15 = 19.7 \text{ U}$ (to three significant figures). Using eqn [62.1], the same preparation would have an activity, expressed in katal, of: $295 \times 10^{-6} \div 900 = 3.28 \times 10^{-7} = 328 \text{ nkat}$ (to three significant figures).

This is measured in terms of the rate of enzyme reaction. You can express activity as amount of substrate utilised per unit time (for example, nmol min^{-1} , etc.), or in terms of the non-SI international unit (U, or sometimes IU), defined as the amount of enzyme which will convert 1 μmol of substrate to product(s) in 1 min under specified conditions. However, the recommended (SI) unit of enzyme activity is the katal (kat), which is the amount of enzyme that will convert 1 mol of substrate to product(s) in 1 s under optimal conditions, determined from the following equation:

$$\text{enzyme activity(kat)} = \frac{\text{substrate converted (mol)}}{\text{time (s)}} \quad [62.1]$$

This unit is relatively large ($1 \text{ kat} = 6 \times 10^7 \text{ U}$) so you will probably need to use SI prefixes, for example, nkat or pkat (p. 192). Note that the units involve amount of substrate (mol), not concentration (mol L^{-1} or mol m^{-3}).

Example Using eqn [62.4] an increase in specific activity from 10 nkat $(\text{mg protein})^{-1}$ to 120 nkat $(\text{mg protein})^{-1}$ represents a 12-fold purification.

For enzymes with macromolecular substrates of unknown molecular weight (for example, deoxyribonuclease, amylase), you can express activity as the mass of substrate consumed (for example, ng DNA min^{-1}), or amount of product formed (for example, $\text{nmol glucose min}^{-1}$). You must ensure that your units clearly specify the substrate or product used, especially when the enzyme transformations involve different numbers of substrate or product molecules.

Degree of purity

The specific activity of an enzyme preparation expresses the enzyme activity in terms of the quantity of protein present:

$$\text{Specific activity} = \frac{\text{enzyme activity (kat)}}{\text{mass of protein (kg)}} \quad [62.2]$$

Alternative units may be used, for example, U mg^{-1} , $\text{ng min}^{-1} \text{ mg}^{-1}$. The specific activity is a useful way of comparing the purity of different enzyme

Purifying enzymes – this usually involves several stages:

1. **Tissue/cell disruption**, often using mechanical/ultrasonic homogenisation (Chapter 42).
2. **Differential centrifugation**, removing larger particulate components (Chapter 44).
3. **Ammonium sulphate fractionation**: selective precipitation by the stepwise addition of ammonium sulphate at $<10^{\circ}\text{C}$ (Chapter 61). The precipitated protein is then redissolved in a fresh buffer solution.
4. **Chromatographic and/or electrophoretic separation**, including ion exchange and gel filtration chromatography and polyacrylamide gel electrophoresis or isoelectric focusing (Chapters 48–51). This stage may involve several individual steps and can lead to a fully purified enzyme (purification to homogeneity), with maximum specific activity.

Example The hydrolysis of one molecule of maltose to give two glucose molecules by α -glucosidase means that enzyme activity specified in terms of substrate consumption (nmol maltose) would be half the value expressed with respect to product formation (nmol glucose).

Example If an original preparation of 50 mL had an activity of 100 pkat mL^{-1} (total 5 nkat) and a subsequent fraction of 5 mL volume had an activity of 200 pkat mL^{-1} (total 1 nkat), using eqn [69.4] this would represent a percentage yield of 20%.

preparations (purified enzyme preparations have high specific activities), comparing the stages in purification of an enzyme (specific activity increases as other proteins are eliminated), and assessing enzyme stability (an unstable enzyme will show a decrease in specific activity with time). Enzyme activity and purity are often expressed in terms of the protein content of the enzyme preparation. Most assays for protein content do not give an absolute value, but require standard solutions, containing appropriate amounts of a particular protein, to be analysed at the same time, enabling a standard curve to be constructed (Chapter 43). Bovine serum albumin (BSA) is commonly used as a general-purpose protein standard. However, you may need to use an alternative standard if your enzyme has an amino acid composition that is markedly different from BSA, depending on the specific method you use (Box 57.1).

At each step in the procedure, the percentage yield of enzyme in an individual fraction can be determined:

$$\text{yield} = \frac{\text{enzyme in fraction (kat or U)}}{\text{enzyme in original preparation (kat or U)}} \times 100 \quad [62.3]$$

Note that the yield equation does not use a concentration-based measure of enzyme activity, as this would be affected by the volumes of the solutions involved. The relative purification of an enzyme is usually expressed as ‘ n -fold purification’, where:

$$n = \frac{\text{specific activity of fraction}}{\text{specific activity of original preparation}} \quad [62.4]$$

Turnover number

The turnover number of an enzyme is the amount of substrate (mol) converted to product in 1 s by 1 mol of enzyme operating under optimum conditions. In practice, to estimate this parameter, you require information on the molecular weight of the enzyme, the amount of enzyme present and its maximum activity.

Measuring the rates of enzyme reactions – types of assay

The rate of substrate utilisation or product formation must be measured under controlled conditions, using some characteristic which changes in direct proportion to the concentration of the test substance.

Spectrophotometric assays

Many substrates and products absorb visible or UV light, and the change in absorbance at a particular wavelength provides a convenient assay method (Chapter 46). In other cases, a product may be measured by a colorimetric chemical reaction.

Several assays are based on interconversion of the nicotinamide adenine dinucleotide co-enzymes NAD^{+} or NADP^{+} which are reversibly reduced in many enzymic reactions. The reduced form (either NADH or NADPH) can be detected at 340 nm, where the oxidised form has negligible absorbance. An alternative approach is to use a coupled enzyme assay, where a product of the test enzyme is used as a substrate for a second enzyme reaction which involves oxidation/reduction of nucleotide coenzymes. Such assays are particularly useful for continuous monitoring of enzyme activity

Examples

α -Glucosidase activity can be measured using the artificial substrate *p*-nitrophenol- α -D-glucose, which liberates *p*-nitrophenol (yellow) with an absorption maximum at 404 nm.

Phosphoenolpyruvate carboxylase (PEP carboxylase) can be assayed by coupling to malate dehydrogenase (MDH): the PEP carboxylase reaction converts phosphoenolpyruvate to oxaloacetate, which is then oxidised to malate by MDH (present in excess), with a stoichiometric (1 : 1) reduction of NAD⁺. The coupled assay is monitored spectrophotometrically as an increase in A_{340} against time.

Understanding fluorescence – the basis of this phenomenon is explained on p. 342.

and for reactions where the product from the test substance is too low to detect by other methods, since coupled assays are more sensitive. Note that the reaction of interest (test enzyme) must be the rate-limiting process, not the indicator reaction (second enzyme).

Radioisotopic assays

These are useful where the substrate and product can be easily separated, for example, in decarboxylase assays using a ¹⁴C-labelled substrate, where gaseous ¹⁴CO₂ is produced. For measurement methods, see Chapter 54.

Electrochemical assays

Enzyme reactions involving acids and bases can be monitored using a pH electrode (p. 162), though the change in pH will also affect the activity of the test enzyme. An alternative approach is to measure the amount of acid or alkali required to maintain a constant preselected pH in a pH-stat.

An oxygen electrode can be used if O₂ is a substrate or a product (p. 385). Other ion-specific electrodes can monitor ammonia, nitrate, etc.

Measuring the rates of enzyme reactions – using chromogenic and fluorogenic enzyme substrates

Artificial substrates that generate either (i) a coloured product (chromogens) or (ii) a fluorescent product (fluorogens) are used widely for the detection and quantification of enzymes, especially when no suitable spectrophotometric assay is available for the natural product or the natural substrate. In most cases, these artificial substrates are analogues of the natural substrates, composed of a ‘core molecule’ that is either fluorescent (p. 342) or coloured, coupled to another group by a covalent bond. The addition of this group to the core molecule usually either causes a decrease in fluorescence and a shift in the excitation and emission signal to longer wavelengths, or converts the coloured compound to a non-coloured form. The covalent bond between core molecule and added group is recognised and cleaved by the target enzyme to liberate the core molecule, thereby generating either a fluorescent or a coloured product that can be measured fluorimetrically (p. 342) or spectrophotometrically (p. 339). Such substrates serve as a sensitive and specific means of detecting individual enzymes in the presence of a range of other biomolecules. The principle is best illustrated using a specific example of each type of substrate (see Fig. 62.1).

Applications of this approach include:

- **biochemical identification of bacteria**, on the basis of enzyme profiles (see also Fig. 62.1)
- **enzyme-linked immunoassays**, for example, ELISA (p. 396)
- **nucleic acid hybridisation and blotting** (pp. 516–17)
- **detection of specific enzymes** in living cells
- **quantitative assay for enzyme activity** – for example, fluorogenic substrates for HIV protease.

Note that care is required for quantitative fluorimetric assay, since impurities in the enzyme preparation can lead to background fluorescence or to quenching (reduction) of the signal: appropriate controls must be run at the same time as test samples.

Fig. 62.1 (a) The chromogenic substrate *ortho*-nitrophenyl- β -D-galactoside (ONP- β -GAL); (b) the fluorogenic substrate 4-methylumbelliferyl- β -D-glucuronide (MU- β -GUR). Both substrates show minimal colour and/or fluorescence owing to the coupling of the core molecule to a carbohydrate group. ONP- β -GAL is cleaved by the enzyme β -galactosidase to liberate ONP, which is yellow (usually assayed spectrophotometrically at 420 nm). In contrast, the cleavage of MU- β -GUR by the enzyme β -glucuronidase liberates MU, which is strongly fluorescent under UV light (e.g. excitation wavelength 360 nm, emission wavelength 440 nm). These two substrates are used to detect and differentiate between coliform bacteria (which contain β -galactosidase) and *Escherichia coli* (which contains both β -galactosidase and β -glucuronidase), e.g. in the IDEXX Colilert system for water analysis (see: <http://www.idexx.com/water/colilert/>).

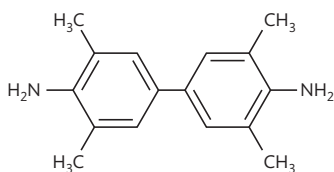
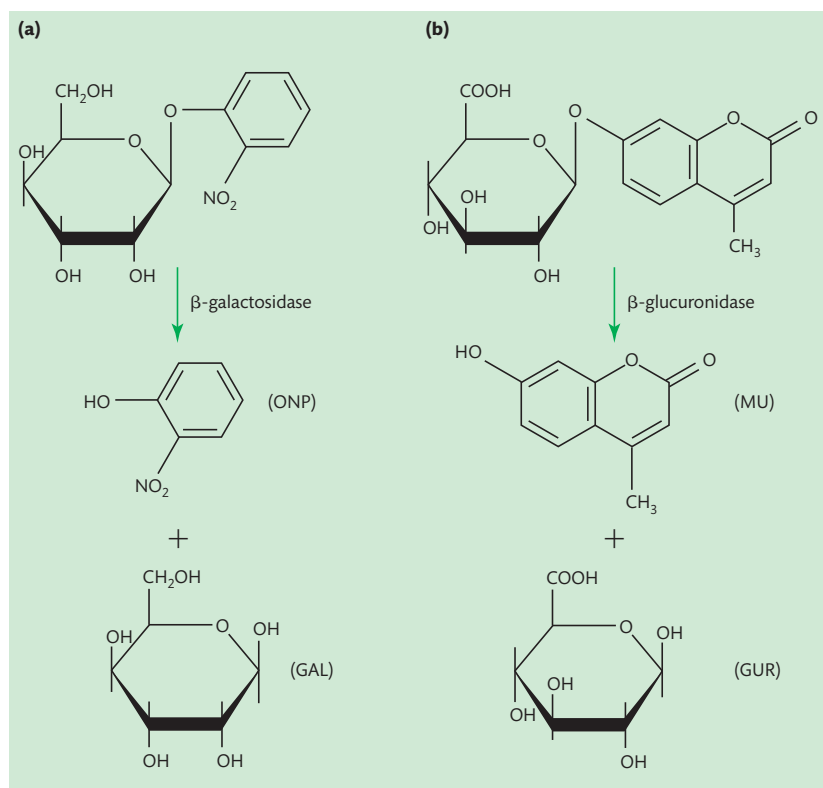


Fig. 62.2 The chromophore 3,3',5,5'-tetramethylbenzidine (TMB), used in peroxide assays, e.g. in ELISA (p. 398).

SAFETY NOTE – TMB is harmful if swallowed or absorbed through the skin – avoid ingestion and skin contact.

Example Detection of enzymes that degrade β -lactam antibiotics in resistant bacteria can be carried out using the chromogenic substrate nitrocefin, which changes from pale yellow to red when hydrolysed by β -lactamases.

A wide range of chromogenic core molecules (chromophores) are used in artificial substrates (Table 62.1), including:

- **nitrophenols** (for example, ONP, Fig. 62.1)
- **nitroanilines** (for example, *Z*-arginine-*para*-nitroanilide derivatives for assaying trypsin activity)
- **indoxyl substrates** (for example, 5-bromo-4-chloro-3-indolyl- β -D-galactoside, also known as X-GAL, which is used widely to detect the activity of the *lacZ* gene in molecular biology, p. 533)
- **alizarin** derivatives and
- **3,3',5,5'-tetramethylbenzidine** (Fig. 62.2).

Similarly, several different fluorophores are commercially available, including:

- **coumarin derivatives** (for example, 4-methylumbelliferone, MU, Fig. 62.1, and 7-amino-4-methylcoumarin, AMC, which fluoresce blue)
- **fluorescein** (Fig. 47.4) and
- **resorufin** (Table 62.1).

For further details see for example, Pala *et al.*, (2020)

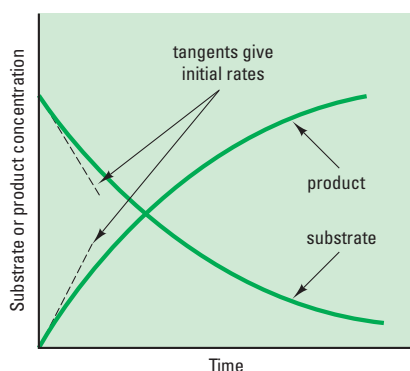
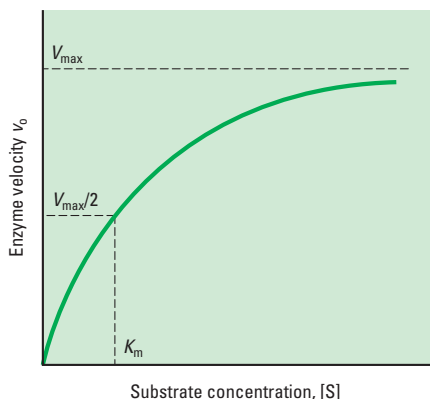
Measuring the rates of enzyme reactions – monitoring substrate utilisation/product formation

Continuous assays (kinetic assays)

Here, you measure the change in substrate or product as a function of time, to provide a progress curve for the reaction (Fig. 62.3). These curves start off in a near-linear manner, decreasing in slope as the reaction proceeds

Table 62.1 Core molecules used in chromogenic and fluorogenic enzyme substrates.

Core molecule	Reaction
Alizarin	Red colour
Aminomethyl coumarin	Blue fluorescence
Fluorescein	Green fluorescence
Indoxyl (and derivatives)	Blue colour (and others)
Methylumbelliferone	Blue fluorescence
Nitroaniline	Yellow colour
Ortho-nitrophenol	Yellow colour
Tetramethylbenzidine	Blue (yellow at low pH)
Rhodamine	Red fluorescence

**Fig. 62.3** Enzyme reaction progress curve: substrate utilisation/product formation as a function of time.**Fig. 62.4** Effect of substrate concentration on enzyme activity.

and substrate is used up. The initial velocity of the reaction (v_0) is obtained by drawing a tangent to the curve at zero time and measuring its slope. Continuous monitoring can be used when the test substance can be assayed rapidly (and non-destructively), for example, using a chromogenic substrate. Reaction rate analysers allow simultaneous addition of reactant(s) or enzyme, mixing and measurement of absorbance: this enables the initial rate to be determined accurately.

Discontinuous assays (fixed time assays)

It is sometimes necessary to measure the amount of substrate consumed or product formed after a fixed time period, for example, where the test substance is assayed by a (destructive) colorimetric chemical method. It is vital that the time period is kept as short as possible, with the change in substrate concentration limited to around 10%, so that the assay is within the linear part of the progress curve (Fig. 62.3). A continuous assay may be carried out as a preliminary step, to determine whether the reaction is approximately linear over the time period to be used in the fixed time assay.

Measuring the rates of enzyme reactions – quantifying enzyme kinetics

For most enzymes, when the initial reaction rate (v_0) using a fixed amount of an enzyme is plotted as a function of the concentration of a single substrate [S] with all other substrates present in excess, a rectangular hyperbola is obtained (Fig. 62.4). At low substrate concentrations v_0 is directly proportional to [S], with a decreasing response as substrate concentration is increased until saturation is achieved. The shape of this plot can be described by a mathematical relationship, known as the Michaelis–Menten equation:

$$v_0 = \frac{V_{\max} [S]}{K_m + [S]} \quad [62.5]$$

This equation makes use of two kinetic constants:

1. **V_{\max} , the maximum velocity of the reaction** (at infinite substrate concentration).
2. **K_m , the Michaelis constant**, is the substrate concentration where $v_0 = \frac{1}{2} V_{\max}$.

V_{\max} is a function of the amount of enzyme and is the appropriate rate to use when determining the specific activity of a purified enzyme. The Michaelis constant is expressed in terms of substrate concentration (mol L^{-1}) and is independent of enzyme concentration. K_m is derived from the individual rate constants of the reaction – for example, with a single substrate (S) and single product (P) enzymic reaction, the process can be described as follows:



where E = enzyme, ES = enzyme-substrate complex, and k_1 , k_2 and k_3 are rate constants. The Michaelis constant can be expressed as:

$$K_m = \frac{k_2 + k_3}{k_1} \quad [62.7]$$

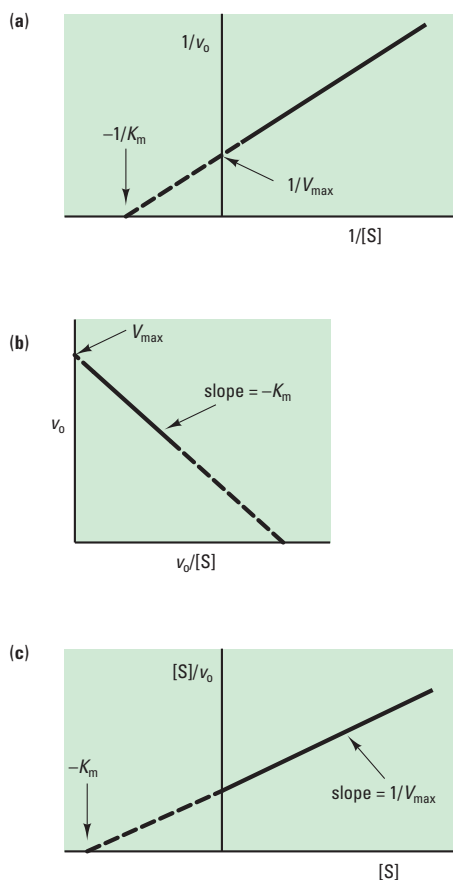


Fig. 62.5 Graphical transformations for determining the kinetic constants of an enzyme. (a) Lineweaver-Burk plot. (b) Eadie-Hofstee plot. (c) Hanes-Woolf plot.

Example The EMBOSS free, open-source software package (at <http://emboss.sourceforge.net/>) can be used to find the K_m and V_{\max} of an enzyme, using Michaelis-Menten and Hanes-Woolf plots.

Removing a bottle of freeze-dried enzyme from a freezer or fridge – do not open until it has been warmed to room temperature or water may condense on the contents – this will make weighing inaccurate and may lead to loss of enzyme activity.

For many enzymes, $k_3 \ll k_2$ and eqn [62.7] simplifies to:

$$K_m = \frac{k_2}{k_1} = \frac{[E][S]}{[ES]} \quad [62.8]$$

When this applies, K_m provides a measure of the affinity of an enzyme for the substrate, and this is an important characteristic of each particular enzyme. Values for mammalian enzymes usually fall within the range $10^{-2} - 10^{-5} \text{ mol L}^{-1}$. Thus an enzyme with a large K_m usually has a low affinity for its substrate, while an enzyme with a small K_m usually has a high affinity. You can use K_m values to select a substrate concentration that will give maximum reaction velocity or to compare the affinities of different substrates for a given enzyme, or the same substrate with different enzymes.

Your first step in determining the kinetic constants for a particular enzyme is to measure the rate of reaction at several substrate concentrations, as in Fig. 62.4. There are various ways to obtain K_m and V_{\max} from such data, mostly involving drawing a graph representing a linear transformation of eqn [62.5].

- **The Lineweaver-Burk plot:** a graph of the reciprocal of the reaction rate ($1/v_0$) against the reciprocal of the substrate concentration ($1/[S]$) gives $-1/K_m$ as the intercept of the x axis and $1/V_{\max}$ as the intercept of the y axis (Fig. 62.5(a)). The slope of the plot is most affected by the least accurate values, i.e. those measured at low substrate concentration.
- **The Eadie-Hofstee plot:** v_0 against $v_0/[S]$, where the intercept on the y axis gives V_{\max} and the slope equals $-K_m$ (Fig. 62.5(b)).
- **The Hanes-Woolf plot:** $[S]/v_0$ against $[S]$, giving $-K_m$ as the intercept of the x axis and $1/V_{\max}$ from the slope (Fig. 62.5(c)).
- **The direct linear plot** (Isenbhal and Cornish) is another option that may give more accurate estimates of kinetic constants than the other plots.

There are several computer packages that you can use to plot the above relationships and calculate the kinetic constants from a given set of data using linear regression analysis (p. 611). While the Eadie-Hofstee and Hanes-Woolf plots distribute the data points more evenly than the Lineweaver-Burk plot, the best approach to such data is to use non-linear regression on untransformed data. This is usually outside the scope of the simpler computer programs, though tailor-made commercial packages can carry out such analyses (for example, the SigmaPlot® enzyme kinetics module). Note also that some enzymes, particularly those involved in the control of metabolism, do not show Michaelis-Menten kinetics.

Taking account of factors affecting enzyme activity

If you want to measure the maximum rate of a particular enzyme reaction, you will need to optimise the following.

Co-factors

Many enzymes require appropriate concentrations of specific co-factors for maximum activity. These are subdivided into co-enzymes (soluble, low molecular weight organic compounds that are actively involved in catalysis by accepting or donating specific chemical groups, i.e. they are co-substrates of the enzyme; examples include NAD^+ and ADP); and activators (inorganic metal ions, required for maximal activity, for example, Mg^{2+} , K^+).

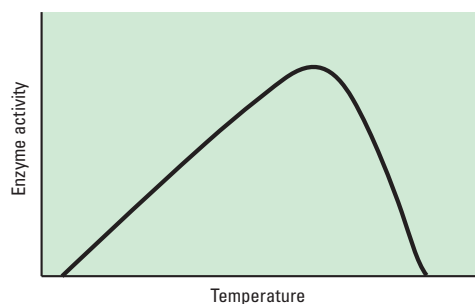


Fig. 62.6 Effect of temperature on enzyme activity.

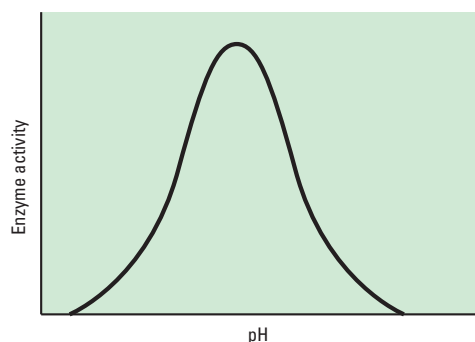


Fig. 62.7 Effect of pH on enzyme activity.

Temperature

Enzyme activity increases with temperature, until an optimum is reached. Above this point, activity decreases as a result of protein denaturation (Fig. 62.6). Note that the optimum temperature for enzyme *activity* may not be the same as that for maximum *stability* (enzymes are usually stored at temperatures near to or below 0 °C, to maximise stability).

pH

Enzymes work best at a particular pH, because of changes in ionisation of the substrates or of the amino acid residues within the enzyme (Fig. 62.7). Most enzyme assays are performed in buffer solutions to prevent changes in pH during the assay. Note that some enzymes have different pH optima for different substrates.

Substrate concentration

A substrate must be present in excess to ensure maximum reaction velocity. The K_m of an enzyme can be used to predict the substrate concentration required for the enzyme to operate at or near its maximum rate – this occurs when all active sites of the enzyme are filled. Using the Michaelis–Menten equation, the fraction of active sites filled (f_{ES}) for a given reaction velocity, v_0 , is given by:

$$f_{ES} = \frac{v_0}{V_{max}} = \frac{[S_0]}{[S_0] + K_m} \quad [62.9]$$

When $[S_0] = K_m$, 50% of active sites are filled, and the reaction proceeds at $\frac{1}{2}V_{max}$. When $[S_0]$ is 10-fold greater than K_m , 91% of active sites are filled. When $[S_0]$ is 100-fold greater than K_m , 99% of active sites are filled and the reaction proceeds at 99% of V_{max} .

KEY POINT Note that enzyme temperature and pH optima are dependent upon reaction conditions, including co-factor and substrate concentrations – you should therefore specify the experimental conditions under which such optima are determined.

Definitions

Inhibition – the reduction in enzyme activity caused by the presence of another compound (an inhibitor).

Negative feedback – a process within a metabolic pathway where the product of a reaction inhibits an enzyme earlier in the pathway, resulting in a reduction in product formation.

Studying enzyme inhibition

It is important to investigate the inhibition of enzyme activity by specific molecules and ions because:

- **enzyme inhibition is an important control mechanism in biological systems** (for example, negative feedback by a product)
- **many drugs act by inhibiting enzymes**
- **the action of many toxins can be explained by enzyme inhibition.**

In terms of their interaction with enzymes, inhibitors can be involved in either reversible or irreversible reactions, and the inhibition can be competitive, non-competitive or uncompetitive, depending on whether inhibition can be reduced by an increase in the concentration of the natural substrate.

Determining the effects of an inhibitor on enzyme kinetics – measure enzyme activity at ≥ 6 different substrate concentrations, both in the presence and absence of inhibitor (see Fig. 62.9).

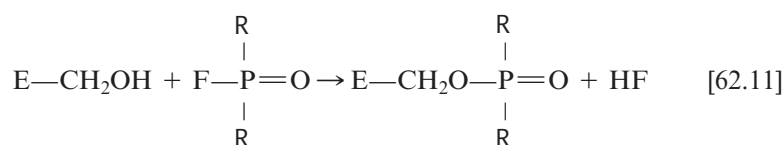
Irreversible inhibitors

These are substances, usually not of biological origin, which react covalently with an enzyme (E), preventing substrate binding or catalysis, for example, iodoacetamide, which binds to thiol groups in enzymes as follows:



The thiol group may be within the active site, forming part of the binding and/or the catalytic sites, or it may be further from the active site and affect the 3D conformation of the enzyme.

The toxicity of heavy metal ions (for example, Hg^{2+}) is largely because of their irreversible effects on enzyme activity. Other examples include the inhibition of acetylcholinesterase by organophosphate pesticides and nerve agents. In such cases, it is often an active site serine residue that is affected, for example, with diisopropylfluorophosphate (DFP):



where R = isopropyl residue.

Reversible inhibitors

These inhibitors do not react covalently with an enzyme, but show rapid reversible binding and dissociation. The velocity of the enzyme-catalysed reaction is reduced by the formation of enzyme–inhibitor (EI) or enzyme–substrate–inhibitor (ESI) complexes. Reversible inhibitors are subdivided according to their effects on K_m and V_{\max} :

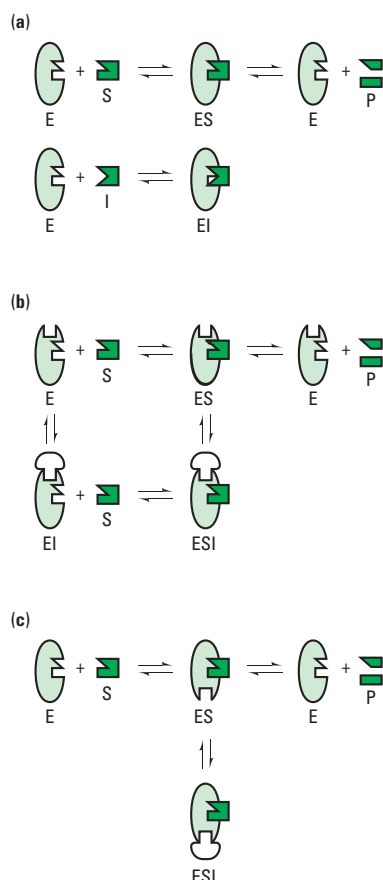


Fig. 62.8 Representation of: (a) competitive inhibition, where the inhibitor (I) binds to the same site on the enzyme (E) as the substrate (S); (b) simple linear non-competitive inhibition; (c) uncompetitive inhibition (P = products).

- **Competitive inhibitors bind reversibly to groups in the active site.** Often the inhibitor resembles the substrate, and the occupation of the active site by an inhibitor molecule prevents a substrate molecule from binding to the same active site (Fig. 62.8(a)). The enzyme (E) can bind to the substrate (S), to form an ES complex, or to the inhibitor (I), to form EI, but cannot bind to both (ESI). A competitive inhibitor lowers the rate of catalysis by reducing the proportion of enzyme molecules bound to the substrate. It is possible to reverse competitive inhibition by increasing the substrate concentration; V_{\max} is unaffected, but K_m increases. An example of competitive inhibition is the inhibition of succinate dehydrogenase by malonate, an analogue of the natural substrate, succinate.
- **Non-competitive reversible inhibition occurs when an inhibitor binds to an enzyme whether or not the active site is occupied by the substrate.** This type of inhibition often involves natural inhibitors of enzymes, and is important in the control of metabolism. The inhibitor binds to a site other than the active site (Fig. 62.8(b)). K_m is unchanged because the affinity of substrate molecules that bind to any uninhibited enzyme is unaffected, but V_{\max} decreases owing to the concentration of active enzyme molecules being effectively reduced by the presence of inhibitor.
- **Uncompetitive inhibition, where the inhibitor binds only after the substrate has bound to the enzyme** (Fig. 62.8(c)). It is most common in reactions involving more than one substrate.

Using enzyme inhibition kinetics to identify the type of inhibitor

You can use enzyme kinetics to distinguish between the different forms of inhibition and to provide quantitative information on the effectiveness of various inhibitors, by allowing the dissociation constant of the enzyme–inhibitor complex, K_i , to be determined. K_i is an expression that relates to the strength of binding of the inhibitor to the enzyme.

Competitive inhibition

With inhibitor present, the enzyme can react with the substrate (eqn [62.3]), but can also react reversibly with the inhibitor (I), to give an inactive enzyme–inhibitor complex (EI), as follows:



As a result, the presence of I decreases the amount of free enzyme [E] available for interaction with S, i.e. $[E] = [E_T] - [ES] - [EI]$, where E_T is the total amount of enzyme present. The concentration of the EI complex depends on the concentration of free inhibitor and on the dissociation constant, K_i , where:

$$K_i = \frac{[E][I]}{[EI]} = \frac{k_2'}{k_1'} \quad [62.13]$$

The Michaelis–Menten equation (eqn [62.5]) must be modified to account for the presence of inhibitor and for a competitive inhibitor, and results in the Lineweaver–Burk plot shown in Fig. 62.9(a). The lines in the presence and absence of inhibitor intersect on the ordinate (y axis), indicating that, with competitive inhibition, the inhibitory effect disappears at high substrate concentration (when $1/[S] = 0$, $[S] = \infty$). The value of K_i can be calculated from knowledge of the K_m values obtained in the presence and absence of inhibitor (intercepts on the x axis), and the following relationship applies:

$$\text{slope}_{\text{inhibited}} = \text{slope}_{\text{uninhibited}} \frac{(1 + [I])}{K_i} \quad [62.14]$$

Non-competitive inhibition

In this case, not only can I bind E, but I can also bind to the ES complex to give an inactive ESI complex, i.e. $E + I \rightleftharpoons EI$ and $ES + I \rightleftharpoons ESI$. In the simplest case, the binding of S has no effect on the binding of I, and *vice versa*, so the dissociation constant of ESI is the same as that of ES, i.e. the K_m . A typical Lineweaver–Burk plot for a non-competitive inhibitor is shown in Fig. 62.9(b). This indicates that this type of inhibitor decreases V_{\max} but does not affect K_m . Effectively, this means that the inhibitor removes a certain fraction of active enzyme from operation, no matter what the concentration of substrate. The V_{\max} changes by a factor of $(1 + [I])/K_i$, and K_i can be obtained by comparison of the slopes obtained with the uninhibited enzyme and the inhibited enzyme, as described above for a competitive inhibitor.

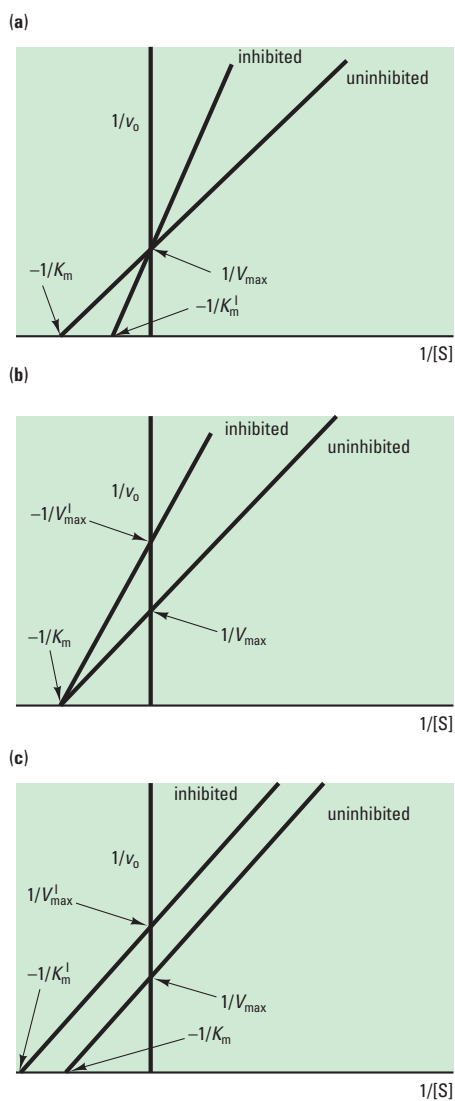


Fig. 62.9 Lineweaver–Burk plots showing the effect of (a) competitive inhibition, (b) simple linear non-competitive inhibition, (c) uncompetitive inhibition.

Uncompetitive inhibition

An uncompetitive inhibitor leads to a Lineweaver–Burk plot as shown in Fig. 62.9(c). Parallel lines are obtained, and both K_m and V_{max} are affected. Here K_i pertains to the dissociation of I from ESI. Uncompetitive inhibitors are fairly rare.

Investigating the control of enzyme activity

In cell metabolism, groups of enzymes work together in sequential pathways to carry out a given metabolic process. In such enzyme systems, the reaction product of the first enzyme becomes the substrate for the next. Most of the enzymes in each system obey Michaelis–Menten kinetics. However, in each system there is at least one enzyme – often the first in the sequence – that sets the rate of the overall sequence (the flux through the pathway) because it catalyses the slowest or rate-limiting step. These regulatory enzymes exhibit increased or decreased catalytic activity in response to certain signals. By the action of such regulatory enzymes, the rate of each metabolic sequence is constantly adjusted to meet changes in the cell's demands for energy and biosynthesis.

The activities of regulatory enzymes are altered by non-covalent binding of various types of signal molecules, which are generally small metabolites or co-factors termed 'modulators'. Such enzymes are generally described as 'allosteric'.

Properties of allosteric enzymes

The following general characteristics enable allosteric enzymes to be identified:

- **Feedback inhibition:** in some multi-enzyme systems the regulatory enzyme is specifically inhibited by the end product of the pathway whenever the end product increases in excess of the cell's needs. The end product does not bind to the active site but binds to another, specific, site termed the regulatory site. This binding is non-covalent and readily reversible. Thus if the concentration of the end product decreases, the rate of enzyme activity increases.
- **Modulators for allosteric enzymes may be either inhibitory or stimulatory.** An activator is often the substrate itself, and regulatory enzymes for which substrate and modulator are identical are called homotropic. When a modulator is a molecule other than the substrate, the enzyme is heterotropic. Some enzymes have two or more modulators.
- **Each enzyme molecule has one or more regulatory or allosteric sites for binding the modulator.** Just as the enzyme's active site is specific for its substrate, the allosteric site is specific for its modulator. Enzymes with several modulators generally have different specific binding sites for each. In homotropic enzymes the active site and regulatory site are the same.
- **These enzymes are generally larger and more complex than simple enzymes and normally consist of two or more subunits.** A useful technique for preliminary determination of subunit structure is SDS-PAGE (Chapter 50).
- **Michaelis–Menten kinetics are not followed.** When v_0 is plotted against $[S]$ a sigmoid saturation curve normally results (Fig. 62.10), rather than

Definition

Allosteric enzyme – an enzyme having more than one shape or conformation induced by the binding of modulators (Greek *alios*, 'other'; *stereos*, 'solid' or 'shape').

Example In the multi-stage conversion of L-threonine into L-isoleucine in bacteria, the first enzyme in the sequence (threonine dehydratase) is inhibited by isoleucine, the final product.

Example Aspartate transcarbamylase, the principal regulatory enzyme in the synthetic pathway for cytidine triphosphate, has six catalytic subunits and six regulatory subunits.

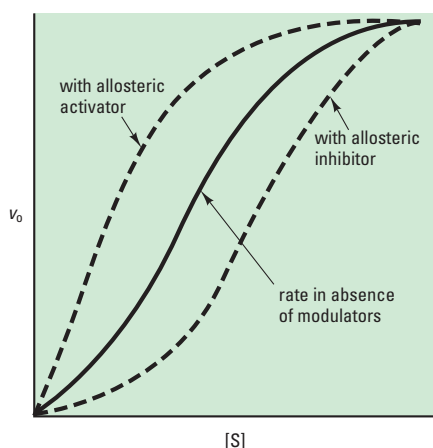


Fig. 62.10 Allosteric effects in enzyme kinetics. The graph shows the rate of enzyme reaction (v_0) against substrate concentration $[S]$.

Example The binding of adrenaline to skeletal muscle receptors triggers reactions that culminate in phosphorylation of a serine residue in glycogen phosphorylase: this activates the enzyme, increasing glycogen breakdown.

the hyperbolic curve shown by non-regulatory enzymes (Fig. 62.4). With sigmoidal kinetics, although a value of $[S]$ can be determined at which v_0 is half maximal, this value is not equivalent to K_m . Instead, the symbol $[S]_{0.5}$, or $K_{0.5}$, is often used to represent the substrate concentration giving half maximum velocity of the reaction catalysed by an allosteric enzyme. Sigmoid kinetic behaviour generally reflects cooperative interactions between multiple protein subunits, i.e. changes in the structure of one subunit result in structural changes in adjacent subunits that affect substrate binding. In general, allosteric activators cause the curve to become more nearly hyperbolic (Fig. 62.10), with a decrease in $K_{0.5}$, but no change in V_{max} , and therefore v_0 is higher for any value of substrate concentration. However, some allosteric enzymes respond to an activator by an increase in V_{max} with little change in $K_{0.5}$. In contrast, an allosteric inhibitor may produce a more sigmoidal curve with an increase in $K_{0.5}$ (Fig. 62.10).

Covalently modified regulatory enzymes

Some enzymes are regulated by covalent modification, for example, by phosphorylation of the hydroxyl groups of specific serine, threonine or tyrosine residues. Depending on the enzyme, this may result in activation or inactivation: in most cases, the non-phosphorylated form of the enzyme is relatively inactive. Often these modifications are under hormonal control. Some regulatory enzymes are influenced both by covalent modification and by allosteric effects.

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STUDY EXERCISES

62.1 Determine enzyme activity and purity (specific activity). An extract of α -glucosidase containing 2.5 mg of protein was incubated with *p*-nitrophenol- α -D-glucose at 1.0 mmol L⁻¹ and assayed by following the liberation of *p*-nitrophenol at 404 nm. This gave a progress curve with an initial velocity equal to an absorbance change of 0.25 min⁻¹. Given that the conversion of 10 nmol of substrate to product results in an absorbance change of 0.122:

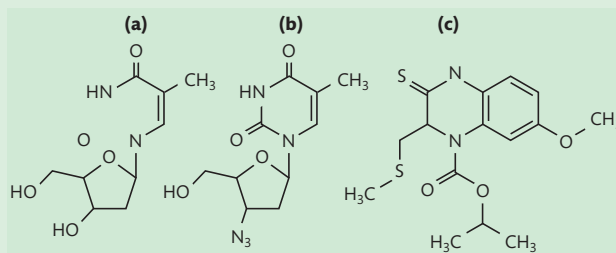
- determine the amount of product formed in nmol min⁻¹;
 - calculate the enzyme activity in kat;
 - determine the specific activity, in terms of (i) nmol min⁻¹(mg protein)⁻¹ and (ii) kat (mg protein)⁻¹.
- (Quote all answers to three significant figures.)

62.2 Estimate K_m and V_{max} for an enzyme. The table below shows representative data for enzyme activity as a function of substrate concentration. Use a suitable graphical method to determine the kinetic constants K_m and V_{max} of the enzyme. (Give your answers to three significant figures.)

Enzyme activity at various substrate concentrations.

Substrate concentration (μmol L ⁻¹)	Enzyme activity (U)
0	0
5	16.2
7	20.5
10	28.1
15	32.7
25	39.2
50	56.7
100	68.2

62.3 Identify the type of enzyme inhibition shown by therapeutic drugs. In the treatment of acquired immunodeficiency syndrome (AIDS), a possible mode of therapy is to inhibit the reverse transcriptase (RT) of the human immunodeficiency virus (HIV), which is required for the retrovirus to be propagated by RNA-directed DNA synthesis. In the figure, one of the substrates for RT is thymidine (a); two drugs, AZT (b) and HBV097 (c) are known to inhibit HIV RT.



Look at the structures and predict the type of inhibition (i.e. competitive or non-competitive) likely to be shown by each drug. Outline an experiment that would enable you to confirm the type of inhibition by investigating enzyme kinetics and explain how you would interpret the results.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

63 Measuring membrane transport

Choosing an experimental system – transport studies can be carried out using whole multicellular organisms (e.g. fish), tissue slices/discs from animals or plants, bacterial cell suspensions or subcellular membrane vesicles, prepared by homogenisation and fractionation (Chapter 41).

All living cells have a variety of processes whereby solutes (substances in aqueous solution) move across membranes, including the uptake of nutrients, the loss of metabolic waste products at the plasma membrane and the intracellular transport of substances across internal membranes. In microbes, the plasma membrane plays the principal role in the selective movement of biomolecules into, or out of, the cell. In multicellular animals and plants, membrane transport processes in the outermost epithelial layers are especially important in determining the solute composition of tissues and organs. Individual organs often have specialised transport functions; for example, in animals, urea excretion via the proximal tubular epithelial cells of the kidney or, in plants, the uptake of nutrients by root hair cells. You are likely to study membrane transport either in ‘hands-on’ lab classes using model systems (for example, dialysis tubing or erythrocytes) or through analysis and interpretation of data for uptake or loss of solutes.

Measuring solute transport

Usually, the rate of movement of a solute in a particular biological system is determined using a radioisotope-labelled (p. 402) or a stable isotope-labelled solute (p. 335) as a ‘tracer’. Typically, in solute uptake (influx), you add the labelled solute to the external medium and then monitor its accumulation by assaying samples of cells or tissue after known time intervals, enabling you to plot the accumulation of tracer as a function of time. You can study loss of solute (efflux) using cells or tissues pre-loaded with labelled solute and then transferred to a tracer-free incubation medium: assaying samples of the medium after known time intervals enables you to follow the time course of efflux without destruction of the material. For cell suspensions, you can use filtration or silicone oil microcentrifugation (p. 321) to separate cells from their incubation medium.

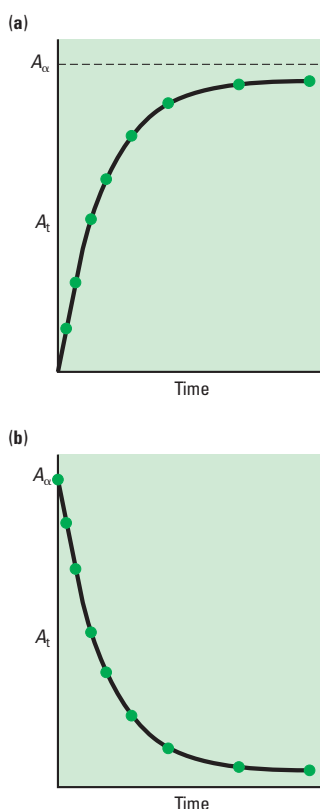


Fig. 63.1 Time course of (a) tracer influx and (b) tracer efflux (A_t). A_α = tracer activity at equilibrium.

KEY POINT Determination of the relationship between the amount of tracer and the amount of solute (the ‘specific activity’, p. 404) enables the uptake or loss of solute to be expressed in terms of the *amount* of solute transported per unit biomass (e.g. per mg protein) per unit time. If the relationship between biomass and membrane area is known, the uptake or loss of solute can be expressed per m^2 membrane surface area per unit time.

Short-term tracer studies will give approximate values for the unidirectional flux rate from the labelled side of the membrane: longer term studies may show more complex kinetics owing to two-way tracer movement or to secondary transport within the cells.

For non-metabolised solutes that are accumulated to particular levels within individual cells, for example inorganic ions such as K^+ , Na^+ , Cl^- , the time course for tracer accumulation or loss will often show exponential kinetics, as the transport process allows the tracer to equilibrate across the cell membrane (Fig. 63.1). Such exponential plots can be transformed to give linear plots using the relationship $\ln(1 - A_t/A_\alpha)$ for influx, where A_α is the activity at equilibrium and A_t is the activity after time = t , while a plot of $\ln A_t/A_\alpha$ should be linear when the cell behaves as a single compartment

Example Suppose 5 mL of a red cell suspension containing 2 mg protein mL⁻¹ accumulated 1050 Bq of radiolabelled solute of specific activity 210 Bq nmol⁻¹ in 8 min. The net rate of solute accumulation over this time period, expressed in nmol mg protein⁻¹ min⁻¹, would be $1050 \div 210 \div 10 \div 8 = 0.0625$ ($\equiv 62.5$ pmol mg protein⁻¹ min⁻¹).

Example For a tissue with a surface area of 24 000 m² per m³, an internal K⁺ concentration of 230 mol m⁻³, and an influx rate constant of 0.000036 s⁻¹, substitution into eqn [63.1] gives an influx of: $(0.000036 \times 230) \div 24\,000 = 3.45 \times 10^{-7}$ mol m⁻² s⁻¹ (345 nmol m⁻² s⁻¹).

Example 3-O-methyl glucose and 2-deoxyglucose are non-metabolised analogues of glucose, while methyl- β -D thiogalactoside is a non-metabolised analogue of lactose.

Definition

Fick's first law of diffusion – each solute diffuses in a direction that eliminates its concentration gradient and at a rate proportional to the size of the gradient.

Example For a solute with a flux of 9×10^{-6} mol m⁻² s⁻¹, an internal concentration of 250 mol m⁻³ and an external concentration of 15 mol m⁻³, rearranging eqn [63.2] gives $P = (9 \times 10^{-6}) \div (250 - 15) = 3.83 \times 10^{-8}$ m s⁻¹ (to three significant figures).

with respect to tracer exchange. The slope of such a plot gives a rate constant for exchange (k) that can be used to quantify the unidirectional solute movement, either influx or efflux (f) from the following equation:

$$\phi = \frac{k[C_i]}{M_a} \quad [63.1]$$

where $[C_i]$ is the internal solute concentration, determined either from tracer equilibration or by chemical assay and M_a is the membrane surface area, expressed per unit volume. For some cells and tissues, an initial, short-term component is observed, owing to tracer exchange between the extracellular space and the bulk medium – this can be subtracted from the longer-term component, allowing correction for extracellular tracer content.

Metabolised solutes introduce additional complexity, since they do not simply equilibrate across membranes – for example, glucose may be used as a source of carbon or energy once transported into a cell. Transport studies of metabolised solutes may need to account for such processes, for example by separating and assaying the various labelled metabolites. A simpler alternative is to use a non-metabolised analogue of the solute, enabling you to study transmembrane movement in the absence of metabolism and simplifying the interpretation of tracer studies.

Identifying the transport mechanism for a particular solute

Many studies are carried out to provide information on the nature of the transport process. Transmembrane solute movement is often subdivided into a number of categories, described below.

Simple diffusion – passive transport

In accordance with Fick's first law of diffusion, an uncharged molecule can move through the lipid bilayer of a membrane with a net flux, J , determined by its lipid solubility and by the difference in concentration (strictly, the difference in activity) across the membrane, according to the relationship:

$$J = P([C_o] - [C_i]) \quad [63.2]$$

where P is the permeability coefficient for the substance, while $[C_o]$ and $[C_i]$ are external and internal solute concentrations, respectively. The permeability coefficients of various solutes correlate with their partition coefficients between non-polar organic solvents and water, reflecting their relative solubility in the lipid phase. In experimental systems the flux rate calculated from eqn [63.2] will apply only to the initial state, since diffusion will reduce the concentration gradient and hence the flux rate.

You can use several different characteristics to recognise simple diffusion, including:

- the measured rates of transport are consistent with the lipid permeabilities of artificial lipid bilayers
- the rate of transport is directly proportional to the concentration gradient (eqn [63.2]), giving a straight-line relationship with no evidence of saturation, in contrast to *all* other transport systems
- movement is insensitive to inhibitors, including structural analogues of the solute, metabolic inhibitors and chemical inactivators

Accounting for the passive diffusion of water (osmosis) – this is a special case, because of the high permeability of biological membranes to water and the high concentration of water on each side of the membrane (see Chapter 23).

- **lack of specificity** – chemically similar solutes with comparable lipid solubilities will show similar rates of transmembrane movement.

Simple diffusion can account for the movement of only a limited number of low M_r , uncharged substances, including O_2 , CO_2 , NH_3 and non-polar hydrocarbons. There is negligible transbilayer diffusion of all other polar and ionic solutes, owing to their hydrophilic, lipophobic nature and to the presence of a surrounding shell of water molecules, preventing movement through the hydrophobic interior of the membrane.

KEY POINT All solutes not transported across membranes by simple diffusion are moved by protein-mediated transporters, often termed ‘permeases’, ‘porters’ or ‘translocators’.

Facilitated diffusion – passive transport

Here, the transmembrane movement is the result of specific membrane proteins (often termed ‘uniporters’). While net movement is energetically ‘downhill’, as in simple diffusion, the rate of movement is more rapid than would be predicted from eqn [63.2], since the individual uniporter molecules act as solute-specific channels across the membrane. Examples include glucose transport across the erythrocyte membrane, maltodextrin transport across the outer membrane of Gram-negative bacteria and the stretch-activated ion channels in stomatal membranes. You can use the following features to identify facilitated diffusion:

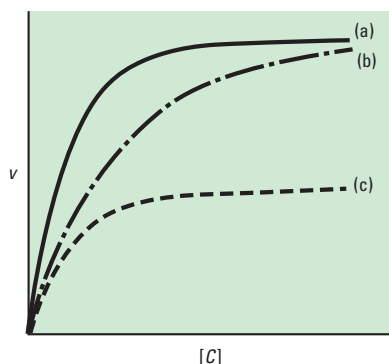


Fig. 63.2 Relationship between the rate of transport (v) and solute concentration $[C]$ for (a) uninhibited ‘control’, (b) with a competitive inhibitor, (c) with a non-competitive inhibitor.

- **The measured rates of transport are far greater than can be accounted for by simple diffusion and Fick’s first law:** for example, the erythrocyte glucose system operates at more than 10 000 times the rate predicted from permeability coefficients of synthetic lipid bilayers.
- **The system exhibits specificity,** transporting the solute but not chemically related compounds with a different 3D shape.
- **The rate of transport should show saturation kinetics similar to those observed for enzymes (Fig. 63.2),** with a hyperbolic relationship between the rate of transport (v) and solute concentration $[C]$ according to the relationship:

$$v = \frac{V_{\max}[C]}{K_s + [C]} \quad [63.3]$$

where V_{\max} is the maximum rate of transport and K_s the half saturation constant, is a measure of the affinity of the transport system for the solute – a low K_s generally indicates a high-affinity transport system while a high K_s shows that the transport system has a low affinity for the solute.

- **Movement is sensitive to competitive inhibitors** – the presence of structurally similar compounds will reduce the rate of solute transport. Competitive inhibitors decrease the affinity of the permease (increased K_s), but V_{\max} is not affected (Fig. 63.2).
- **Transport is susceptible to inactivation by non-competitive inhibitors that act as protein denaturants, for example heavy metal salts.** Such irreversible inactivation confirms that the transport process is mediated

Determining kinetic parameters in transport studies – since eqn [63.3] is equivalent to the Michaelis–Menten equation, the same approaches can be used to determine V_{\max} and K_s , based on measurements of the rate of transport at several different solute concentrations.

by a protein. At low concentrations of inhibitor, only some of the permease molecules will be inactivated, giving a decreased V_{\max} but unchanged K_s (Fig. 63.2).

If facilitated diffusion of an uncharged solute occurs, then net solute movement will occur *down* the transmembrane concentration gradient. However, for charged solutes (ions), net movement will depend on the transmembrane electrical potential (the membrane potential), as well as the concentration gradient. The transmembrane equilibrium potential for a particular ion, E_n , can be calculated from the Nernst equation, as:

$$E_n = 2.303 \frac{RT}{zF} \log \frac{[C_o]}{[C_i]} \quad [63.4]$$

Example For K^+ (a monovalent cation) in a cell with an internal concentration of 450 mol m^{-3} in an experimental solution at an external concentration of 10 mol m^{-3} , substitution into eqn [63.4] with a value for the gas constant, $R = 8.31443 \text{ J K}^{-1} \text{ mol}^{-1}$ and Faraday's constant, $F = 9.648675 \times 10^4 \text{ J V}^{-1} \text{ mol}^{-1}$ at 20°C ($T = 293.15 \text{ K}$) gives a transmembrane equilibrium potential, $E_n = 2.303 [(8.31443 \times 293.15) \div (+1 \times 9.648675 \times 10^4)] \log (10 \div 450) = -0.0961782 \text{ V}$ (-96.2 mV to three significant figures).

where all symbols have their usual meanings (see also Chapter 52). You can compare the equilibrium potential of the ion with the membrane potential to see whether the ion is in electrochemical equilibrium across the membrane, or whether it has been transported against its electrochemical potential gradient.

KEY POINT The transmembrane electrical potential, responsible for the facilitated diffusion of ions, is usually established as a direct result of the action of one or more ion-pumping, active transport systems, e.g. H^+ -ATPases.

Example A membrane potential of -60 mV (interior negative) can account for a tenfold passive accumulation of a monovalent cation and a tenfold passive exclusion of a monovalent anion.

Ionophores (Table 63.1) can be viewed as simple models of facilitated diffusion systems: they can be used to study the effects of an increase in the permeability of membranes to a single solute or group of solutes, to create transmembrane movements of ions, or to monitor specific ions in solution.

Active transport

The principal criterion to establish active transport is that movement of the solute occurs against its electrochemical potential gradient. The energy for such 'uphill' transport can be provided by one of two mechanisms:

Measuring membrane potentials – depending on the cell type, this can be carried out using microelectrodes (pp. 476–7), lipophilic cations or fluorescent dyes.

1. Primary active transport. The movement of solute molecules is coupled to the hydrolysis of ATP, via a membrane-bound solute-specific ATPase, or to a redox chain. For example, the Na^+-K^+ ATPase of mammalian cells is a primary active transporter, moving three Na^+ ions outwards and two K^+ ions inwards for every molecule of ATP hydrolysed, i.e. it is *electrogenic*, with a net charge separation across the membrane. Some of the most important primary active transport processes are those which create a transmembrane H^+ gradient, or proton-motive force (Δp), either via an H^+ -translocating ATPase or an electron transport chain. In many membranes, Δp provides the major driving force for the movement of other solutes (Nicholls and Ferguson, 2013). Chapter 64 gives details of its measurement in organelles; similar techniques can be used with cell suspensions. Some Gram-negative bacteria use a variation of phosphoryl-driven active transport to accumulate certain sugars – this

Table 63.1 Ionophores and inhibitors of transmembrane solute movement*.

Ionophores	Effect
valinomycin	electrogenic carrier: increased permeability to K^+ and Rb^+
gramicidin A	electrogenic channel: uniport of monovalent ions, particularly H^+
monensin	electroneutral carrier: exchange of Na^+ or Li^+ for H^+
nigericin	electroneutral carrier: exchange of K^+ or Rb^+ for H^+
A-23187	electroneutral carrier: exchange of divalent cations (e.g. Ca^{++} , Mg^{++}) for $2H^+$
2,4-dinitrophenol (DNP)	electrogenic carrier: increased H^+ permeability (protonophore)
carbonylcyanide <i>m</i> -chlorophenyl-hydrazone (CCCP)	electrogenic carrier: increased H^+ permeability (protonophore)
carbonylcyanide <i>p</i> -trifluoromethoxy-phenylhydrazone (FCCP)	electrogenic carrier: increased H^+ permeability (protonophore)
nonactin	increased permeability of monovalent cations
amphotericin B	anion channel (weak selectivity)
nystatin	anion channel (weak selectivity)
Transport inhibitors†	Effect
oligomycin	inhibition of F_0F_1 -type H^+ -ATPases (mitochondria, bacteria, chloroplasts)
ouabain	inhibition of Na^+-K^+ -ATPases (mammalian cell plasma membrane)
digitoxigenin	inhibition of Na^+-K^+ -ATPases (mammalian cell plasma membrane)
vanadate	inhibition of E_1E_2 -type ATPases (plant plasma membrane)
<i>N,N'</i> -dicyclohexylcarbodiimide (DCCD)	inhibition of F_0F_1 and V-type ATPases (mitochondria and plant tonoplast)
<i>N</i> -ethylmaleimide (NEM)	inhibition of plant tonoplast V-type ATPases
phlorizin	inhibition of Na^+ -glucose symporters (intestinal epithelial cells)
furosemide	inhibition of co-transporters (mammalian cell membrane)
cytochalasin B	inhibition of glucose transporters (mammalian cell membrane)
amiloride	inhibition of Na^+/H^+ antiporters (mammalian and plant cells)
4,4'-diisothiocyano-2,2'-stilbene-disulfonate (DIDS)	inhibition of mammalian anion antiporters

*Safety note: all of these inhibitors are highly toxic and must be handled carefully, observing the appropriate safety precautions.

†Details of electron transport inhibitors are given in Chapter 64.

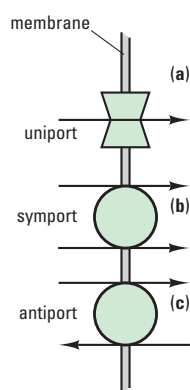


Fig. 63.3 Diagrammatic representation of various membrane transport systems, indicating the direction of movement of transported solute molecules.

is known as ‘group translocation’ since it involves the simultaneous phosphorylation of each sugar molecule as it is transported. Because the cell membrane is impermeable to sugar phosphates, the bacterial ‘phosphotransferase’ system can concentrate these solutes within cells.

- 2. Secondary active transport, or secondary transport.** Here, the movement of solute is coupled to the ‘downhill’ movement of another solute, either via a ‘symporter’ (co-transporter) that simultaneously transports the two solutes in the same direction, or an ‘antiporter’ (counter-transporter) that simultaneously moves the two solutes in opposite directions across the membrane (Fig. 63.3). These systems are ‘active’ only in the sense that the ‘downhill’ movement of the other solute can only occur after primary active transport has established a transmembrane solute gradient (for example, for H^+ or Na^+). Thus, lactose uptake in bacterial cells is coupled to the transmembrane proton gradient, via the lactose- H^+ symporter, while Na^+ efflux from plant cells is mediated by an Na^+-H^+ antiporter. In both instances, the H^+ gradient is created by a primary active transport system that pumps protons out of the cell, generating a proton-motive force that can be utilised by secondary transport systems. Similarly, the Na^+-K^+ primary active transport system creates an Na^+ gradient that can be utilised for nutrient uptake in mammalian cells, for example via the Na^+ -glucose symporter (a secondary transporter). Primary and secondary transport systems can be envisaged as operating as a ‘circuit’ for the movement of ions across membranes (Fig. 63.3).

Definition

Proton-motive force – an electrochemical gradient of protons (H^+) generated by active transport of H^+ across a membrane: the gradient can then be used to carry out work, e.g. solute transport or ATP synthesis.

Interpreting inhibitor experiments – while short-term effects may be unique to a particular inhibitor, in the longer term more general, non-specific effects, are likely. For example, inhibitors of primary active transport will eventually affect secondary transport systems, owing to their effects on ion gradients and membrane potentials.

In addition to the characteristics of specificity, saturation and inhibition shared by facilitated diffusion, you can identify active transport systems based on the following features:

- **The transmembrane distribution of the solute is not consistent with eqn [63.2]** (where $J = 0$ at equilibrium, i.e. $[C_o] = [C_i]$) for an uncharged solute, or eqn [63.4] for an ion.
- **The active component will be sensitive to metabolic inhibitors**, e.g. ATPase inhibitors for primary active transport, or specific inhibitors of secondary transporters (see Table 63.1).
- **For secondary transport systems, dissipation of the primary ion gradient using a suitable ionophore will inhibit transport of the solute.** Conversely, artificially generated ion gradients can be used to drive solute movement, either in whole cells or in membrane vesicle preparations.

Studying membrane transport using microelectrodes

These electrodes have very small tip diameters (typically $\leq 1\mu m$), enabling them to be either (i) inserted through a cell membrane to record intracellular variables, such as pH (Chapter 24), transmembrane variables, such as electrical potential ('membrane potential') and transmembrane ion gradients or (ii) pressed tightly against a cell membrane to record ion currents (Fig. 63.4). Microelectrodes for measuring these variables can be made by heating and pulling narrow-bore glass tubing to create 'micropipettes' – these are filled with KCl and a silver/silver chloride electrode (pp. 143–4) is inserted to create the 'sensing' (recording) microelectrode. The microelectrode is held in a micromanipulator and viewed using a binocular microscope.

Measuring membrane potential

Here, the microelectrode tip is moved up to and through the plasma membrane, which 'seals' around the microelectrode at the site of impalement, due to the fluid properties of the phospholipid bilayer, placing the tip inside the plasma membrane, without bursting the cell (Fig. 63.4). The electrical potential of the impaled cell is then measured against a reference electrode

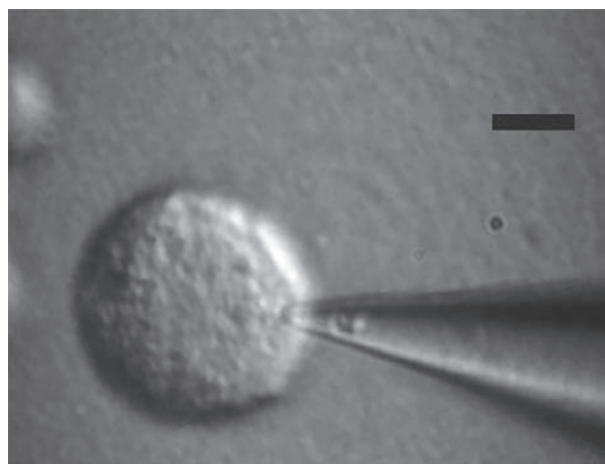


Fig. 63.4 Human mesenchymal stem cell and microelectrode. The scale bar = $10\mu m$ (from Pennisi, C.P., Zachar, V. and Yoshida, K. (2007), reproduced with permission).

Using a micromanipulator – this is a device that translates manual movement (e.g. with a joystick) into scaled-down and damped movements, enabling it to be used for fine control of apparatus at a microscopic level.

connected to the external solution, determined using a millivoltmeter (Fig. 52.2). Typically, cell membrane potentials range from -40 mV to -100 mV (interior-negative). Connecting the output to a recording device allows you to follow changes in membrane potential as a result of experimental treatments (Fig. 63.5).

Ion-selective microelectrodes make use of ion-selective glass at the microelectrode tip – the use of H^+ -specific glass microelectrodes to measure intracellular pH is covered in Chapter 24 (see Fig. 24.5, p. 166). Similar principles apply to the measurement of intracellular Na^+ and K^+ , and their transmembrane gradients (Chapter 52).

Patch clamping

In this relatively advanced technique, part of a membrane (a ‘patch’) is sealed to the end of a heat-polished micropipette of diameter $\cong 1 \mu m$ and the flow of current across the membrane patch is then measured for different bathing solutions and/or transmembrane voltages, set at particular values (‘clamped’) by a feedback amplifier. The size and orientation of

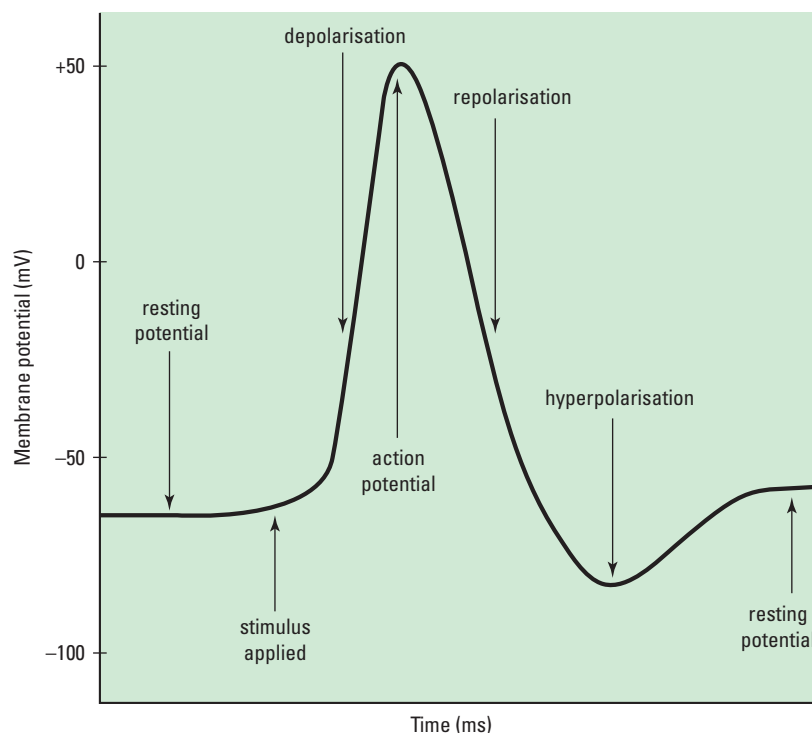


Fig. 63.5 Representation of a recording of the membrane potential of a cell impaled with a microelectrode. The time course shows an action potential (depolarisation, then repolarisation) in response to an applied stimulus, followed by a brief hyperpolarisation, before returning to its original value. Note the negative scale of the Y axis. This trace might be typical of a nerve cell, where relevant stimuli in sensory neurons could include light, sound, temperature or pressure. Such action potentials may move along an axon, where they may cause similar pulses in connected cells leading eventually to responses distant to the original activation. Note that actual recordings of impaled cells will be less smooth than this representation, due to background electrical ‘noise’.

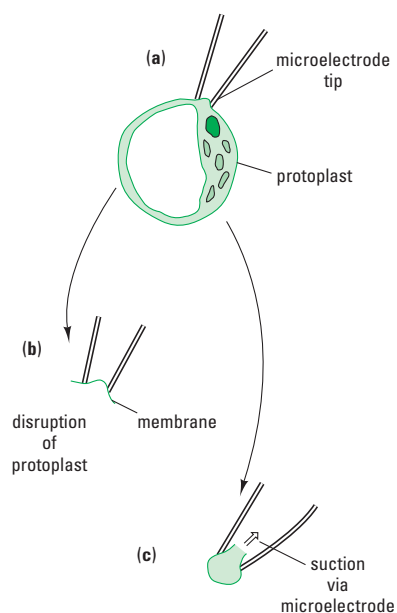


Fig. 63.6 Patch-clamp techniques: (a) whole plant cell, (b) inside-out membrane patch, (c) outside-out membrane patch.

the membrane patch can be controlled, as shown in Fig. 63.6. Since the membrane patch can be relatively small, the operation of ion-transporting proteins can be seen as changes in current flow, owing to the opening or closing of individual membrane ‘channels’ (voltage-sensitive transporters). While the physiological factors controlling the *in vivo* operation of such channels remain unclear, patch clamping provides a powerful means of studying channel-mediated ion movements *in vitro*, enabling investigation of:

- the voltage required to produce ‘channel’ opening
- the membrane conductance under conditions where the ion concentrations on both sides of the membrane are known
- temporal effects, including opening and closing times, frequency of opening, etc.
- the ion selectivity of particular ‘channels’
- the effects of inhibitors, ionophores, etc.

When whole cells are used, the flow of ions through primary active ion transport systems (ion ‘pumps’) can be studied, since changes in current flow represent the sum of many transporters, acting together. Patch-clamp systems have provided a considerable amount of information on the transport properties of individual membranes, for example the plasma membrane and tonoplast of plant cells (see Yeo and Flowers, 2007, for details).

Text references and sources for further study

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STUDY EXERCISES

63.1 Calculate flux rates of various solutes based on data from tracer studies. Using the following data for rate constant (k), internal concentration ($[C_i]$) and membrane surface area (M_a), calculate the unidirectional flux of each solute (express your answers to three significant figures, as for all of the study exercises in this chapter).

- Solute uptake for a cell where $k = 0.0001 \text{ s}^{-1}$, $[C_i] = 150 \text{ mol m}^{-3}$ and $M_a = 50\,000 \text{ m}^2 \text{ m}^{-3}$.
- K^+ efflux from a protozoan, where $k = -1.65 \times 10^{-4} \text{ s}^{-1}$, $[C_i] = 106 \text{ mol m}^{-3}$ and $M_a = 8.25 \times 10^4 \text{ m}^2 \text{ m}^{-3}$.
- Na^+ uptake into a bacterial cell, where $k = 4.75 \times 10^{-5} \text{ s}^{-1}$, $[C_i] = 10.5 \text{ mmol dm}^{-3}$ and $M_a = 1.66 \times 10^{-9} \text{ m}^2 \text{ dm}^{-3}$.

63.2 Determine passive transmembrane fluxes of uncharged solutes. What is the direction and magnitude of the net passive transmembrane flux for each of the following cases, all involving uncharged solutes?

- Movement of glycerol in a halotolerant bacterial cell with an internal concentration of 10 mol m^{-3} , an external concentration of 1.5 mol m^{-3} and a permeability coefficient of $2 \times 10^{-7} \text{ m s}^{-1}$.
- Glucose movement in an animal cell with an internal concentration of 8.65 mol m^{-3} , an external concentration of 0.03 mol m^{-3} and a permeability coefficient of $1.6 \times 10^{-9} \text{ m s}^{-1}$.
- Methanol flow in a fungal cell with an internal concentration of $0.25 \text{ mmol dm}^{-3}$, an external concentration of $3.45 \text{ mmol dm}^{-3}$ and a permeability coefficient of $2.6 \times 10^{-3} \text{ cm s}^{-1}$.

63.4 Interpret kinetic parameters of transport systems.

Details of the kinetic parameters for the transport of an amino acid into cells of three different types are given in the table.

Kinetic parameters for amino acid uptake into three different cell types.

Cell type	V_{\max}	K_s
A	$15.2 \text{ pmol dm}^{-3} \text{ s}^{-1}$	$1.24 \times 10^{-6} \text{ pmol dm}^{-3}$
B	$9.6 \text{ nmol m}^{-3} \text{ s}^{-1}$	$6.54 \times 10^5 \text{ nmol m}^{-3}$
C	$84 \times 10^{-11} \text{ mol m}^{-3} \text{ s}^{-1}$	$2.54 \times 10^{-4} \text{ mol m}^{-3}$

- Which cell type has the highest maximum rate of transport?
- Which cell type has the highest affinity for the amino acid?
- Which cell type gives the fastest rate of uptake at an amino acid concentration of $1 \times 10^4 \text{ mol m}^{-3}$?

63.4 Compare calculated transmembrane equilibrium potentials with the membrane potential to decide whether an ion has been actively transported.

At 25°C , a marine algal cell has a measured plasma membrane potential of -56 mV and the following internal (cytoplasmic) and external ion concentrations.

Internal and external ion concentrations for a marine alga.

Ion	Internal concentration (mol m^{-3})	External concentration (mol m^{-3})
K^+	197	10
Na^+	8.4	225
Cl^-	27.3	235

- What is the transmembrane equilibrium potential for each of these ions?
- Which ions appear to be in electrochemical equilibrium and which appear to be actively transported (and in what direction)?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

64 Quantifying photosynthesis and respiration

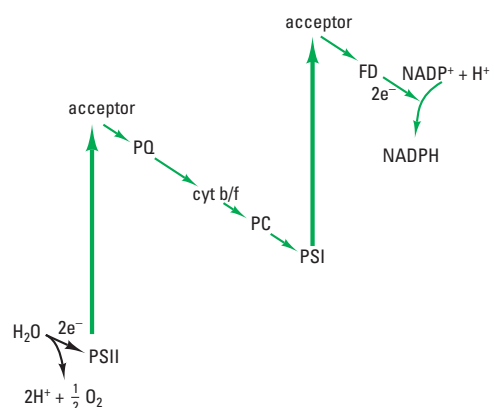


Fig. 64.1 Non-cyclic electron transport from H_2O to NADP^+ in plant photosynthesis (Z scheme) showing the principal components: PS II, photosystem II; PQ, plastoquinone; cyt, cytochrome; PC, plastocyanin; PS I, photosystem I; FD, ferredoxin; NADP^+ , nicotinamide adenine dinucleotide phosphate.

Measuring photosynthetic pigments – equations for spectrophotometric analysis of chlorophylls are given on p. 340.

Definitions

C3 plants – those in which phosphoglyceric acid is the first stable product.

C4 plants – those in which malate or aspartate are the principal products, via oxaloacetic acid, in a reaction catalysed by phosphoenolpyruvate (PEP) carboxylase.

CAM plants – those in which malic acid is produced, via oxaloacetic acid, by PEP carboxylase activity at night, with little or no net assimilation of CO_2 during the day (crassulacean acid metabolism).

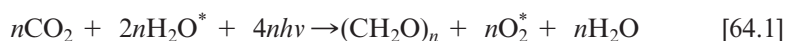
Techniques for investigating photosynthesis and respiration are considered together, since:

- **Gas exchange processes in these two processes are generally studied using similar techniques**, and interpreted in similar stoichiometric terms, for example, by relating changes in O_2 or CO_2 to those of other reactants or products.
- **Membrane-bound electron transport systems are involved in the production of a transmembrane H^+ gradient** that drives ATP synthesis via an ATPase embedded in the same membrane. Measurement of electron transport in both photosynthesis and respiration often makes use of redox dyes as artificial electron donors or acceptors, with spectrophotometric analysis of the reactions.
- **Some electron transport inhibitors can affect both photosynthesis and respiration**, while others are specific to one or the other process.

KEY POINT Photosynthesis and respiration are complex, multistage metabolic pathways involving a large number of cellular components: they may be studied as a whole, or as isolated components and individual metabolic reactions, to gain a deeper insight into the underlying processes.

Quantifying photosynthesis

This is the conversion of light energy to chemical energy by photoautotrophic plants and bacteria. The overall process is often summarised in terms of the synthesis of carbohydrate, $(\text{CH}_2\text{O})_n$, driven by the energy within photons of light ($h\nu$), as:



where the asterisks show that all of the O_2 generated in this process is derived from the photolysis of water. Carbohydrate synthesis occurs as a result of two distinct processes, namely (i) light reactions and (ii) dark reactions. In the former, light energy is absorbed by pigments within membrane-bound reaction centres (photosystems). The water-splitting light reactions generate electrons, protons and molecular oxygen. Electron and proton movement via a series of membrane-bound redox carriers leads to the reduction of NADP^+ and the generation of ATP (photophosphorylation), driven by a transmembrane H^+ gradient, or proton-motive force (p. 488). The light reactions of plant-type photosynthesis are often represented by the ‘Z scheme’ (Fig. 64.1).

The dark reactions use the products of the light reactions to ‘fix’ CO_2 , via a series of soluble enzymes known as the Calvin cycle, or reductive pentose phosphate cycle. The primary carboxylating enzyme in temperate green plants (C_3 plants) and cyanobacteria is ribulose biphosphate carboxylase (‘Rubisco’). This enzyme catalyses the addition of CO_2 to ribulose biphosphate (C_5), producing two molecules of phosphoglyceric acid (C_3) which are then reduced to triose phosphate (C_3) by enzymes that utilise the NADPH and ATP generated by the light reactions. Thus both light and dark reactions operate in a coupled manner when photosynthetically active cells are illuminated with visible light.

Definitions

Gross photosynthesis – the rate of CO_2 uptake or O_2 production, allowing for gas exchange due to respiratory and photorespiratory activity.

Net photosynthesis – the net rate of CO_2 uptake or O_2 production, including respiratory and photorespiratory gas exchange.

Correcting for photorespiration and respiration – measuring the rate of O_2 consumption or CO_2 production in the first few minutes after the plant material has been transferred from the light to darkness will give you the best estimate of photorespiratory and respiratory gas exchange. Use this rate to convert net photosynthesis to gross photosynthesis.

Preventing oxygen supersaturation in oxygen electrode studies – lower the O_2 content of your experimental solutions by bubbling them with N_2 before use, so that the O_2 evolved during the experiment remains in solution.



Fig. 64.2 A portable infrared gas analyser, used to measure photosynthetic CO_2 uptake (leaf cuvette/probe shown in left hand, with analysis unit and user interface below).

Courtesy of PP Systems, Amesbury, MA, USA (<http://www.ppsystems.com>).

Measuring photosynthetic activity

Equation [64.1] shows that the rate of photosynthesis can be measured in terms of the amount of O_2 evolved or CO_2 fixed. However, several other metabolic processes may lead to concurrent changes in O_2 and/or CO_2 status, particularly:

- **Photorespiration** – light-dependent O_2 uptake, due to the oxygenase activity of Rubisco, leading to the production of glycolic acid (C_2) and the subsequent release of CO_2 due to the operation of a ‘scavenging’ pathway where two molecules of glycolate are converted to one of phosphoglyceric acid (C_3). The relative rates of photosynthesis and photorespiration depend upon the concentration of O_2 and CO_2 at the active site of Rubisco – in C_3 plants, photorespiration is highest under conditions of high temperature, high light and low water availability, while C_4 plants show low levels of photorespiration.
- **Respiration** – generation of ATP via carbohydrate oxidation, as in non-photosynthetic cells. In most photoautotrophs, respiratory activity is likely to be lower during the day than at night. However, plant tissues often respond to wounding or environmental stress by *increasing* their rate of respiration – this may be significant if you are measuring photosynthetic activity in tissue fragments, isolated cells or protoplasts. If you wish to calculate gross rates of photosynthesis, you must make appropriate allowances for both respiration and photorespiration.

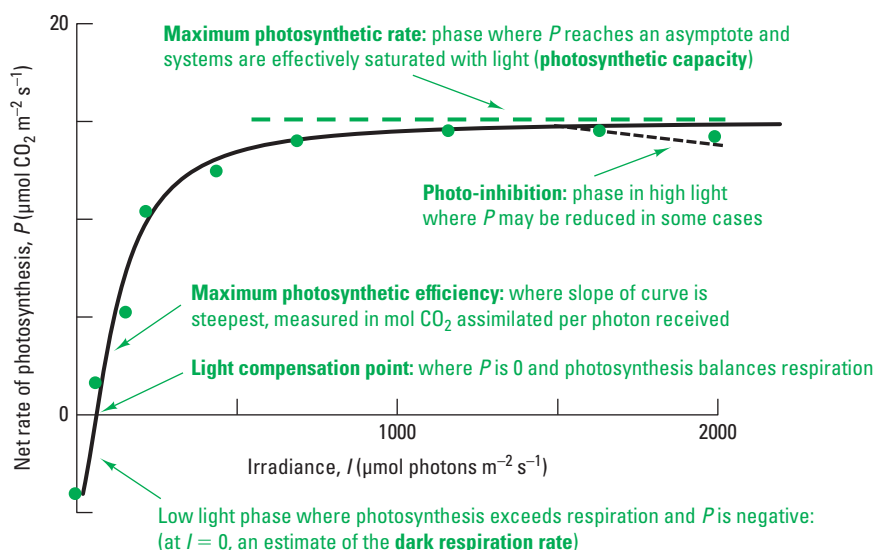
Measuring oxygen production

This is most often used with aquatic systems, including algae and photosynthetic bacteria, higher plant cells, protoplasts, chloroplast suspensions or isolated thylakoids. Oxygen in solution can be determined by end-point chemical analysis (for example, the Winkler method, p. 146) or, more conveniently, by continuous monitoring using an oxygen electrode and chart recorder, to give the rate of net O_2 production (Chapter 52) under various conditions, for example, light, temperature, etc. For photosynthesis–irradiance (P – I) curves using an O_2 electrode, you should note that the electrode assembly can act as a lens, so the light within the chamber can be higher than that measured at the outside surface. The rate of gross photosynthesis is obtained by correcting for O_2 uptake when the electrode assembly is transferred to darkness (for example, using a thick black cloth).

Measuring carbon dioxide uptake

For higher plant studies, this is most easily achieved using an infrared gas analyser, or IRGA (Fig. 64.2). Most modern instruments are portable and generally incorporate: (i) a cuvette, with a transparent window that attaches to a whole leaf or a known area of leaf providing a gas-tight seal, and within which the air is stirred by a fan; (ii) a gas supply system that allows control over input gases; (iii) miniaturised infrared analysers to detect differences in CO_2 and H_2O content of input and output gas streams; (iv) systems for measuring (and possibly controlling) other environmental variables, for example, light and temperature; (v) an on-board microprocessor for calculating results as rates of photosynthesis and transpiration and estimating the leaf’s internal $p\text{CO}_2$ (C_i) and for exporting these and other data (for example, time, leaf temperature, photo-synthetic photon flux density) to PCs. Portable IRGAs are particularly useful for the rapid

Fig. 64.3 Example of a photosynthesis–irradiance (P – I) curve for a typical plant leaf. Such a curve might be obtained using an IRGA, manipulating I in steps down from high values and measuring P rates when stable (see Parsons *et al.*, 1997). Typical values for estimated parameters might be: dark respiration rate, $-4 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; light compensation point, $30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$; maximum photosynthetic efficiency, 8 photons per CO_2 molecule fixed (the theoretical maximum is about 20); maximum photosynthetic rate, $20 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, which might be achieved at $I = 1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (full sunlight is about $1800 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Various methods are used to fit lines to data points and thereby estimate the above parameters – there remains controversy as to which is the best mathematical function to use for this.



Definitions

Compensation point – PFD or $p\text{CO}_2$ at which net photosynthesis = 0, i.e. where gross photosynthesis, respiration and photorespiration are balanced: from the intercept of a P – I or P – C_i curve (x -axis).

Photosynthetic efficiency – the rate of photosynthesis when limited either (i) by supply of photons or (ii) by supply of CO_2 , determined from the initial gradient of a P – I or P – C_i curve respectively.

Photosynthetic capacity – the rate of photosynthesis under saturating photon flux density (PFD) or CO_2 supply, determined from the upper asymptote of a P – I or P – C_i curve respectively.

Interpreting ^{14}C fixation data – short-term studies of a few minutes may estimate gross photosynthesis, while longer-term studies will give a rate closer to net photosynthesis, since some of the fixed ^{14}C will be respired.

Studying the release of photo assimilated ^{14}C in aquatic photoautotrophs – the loss of glycollate and other metabolites can be quantified by acidifying a known amount of medium, to drive off unfixed ^{14}C , then counting directly, or after an appropriate separation procedure.

construction of photosynthesis–irradiance (P – I) and photosynthesis– $p\text{CO}_2$ (P – C_i) curves used to estimate photosynthetic efficiency, photosynthetic capacity and compensation points (Fig. 64.3).

Using carbon isotopes

Radioisotopic ^{14}C and stable ^{13}C are used to study photosynthesis. Radiocarbon (^{14}C) can be supplied as $^{14}\text{CO}_2$ for gas exchange studies or, more readily, as $\text{H}^{14}\text{CO}_3^-$ for studies in aqueous systems, since there will be an interconversion of soluble CO_2 , HCO_3^- and H_2CO_3^* . In aqueous systems, the plant material is incubated in medium containing ^{14}C –labelled bicarbonate for a known time period, then removed and prepared for liquid scintillation counting (pp. 406–7). Microalgal cells and photosynthetic bacteria can be separated from the experimental medium by filtration, or silicone oil microcentrifugation (p. 488) – mild acid treatment (for example, $50 \text{ mmol L}^{-1} \text{ HCl}$) will ensure that any unfixed ^{14}C is released as $^{14}\text{CO}_2$. To express the results in terms of the amount of C assimilated, you will need to calculate (i) the total inorganic C content of the medium, i.e. $\text{CO}_2 + \text{HCO}_3^- + \text{H}_2\text{CO}_3^*$, obtained from pH and alkalinity measurements or by IR spectroscopy of the CO_2 produced when a known amount of the medium is acidified and (ii) the relationship between the total inorganic C content and the amount of ^{14}C tracer added (i.e. the specific activity of the experimental solution).

One of the advantages of studying the photoassimilation of ^{14}C is that the radiotracer can be used to follow the fate of fixed carbon, by separating and fractionating the various cellular components prior to counting, for example, by sequential solvent extraction of the plant material in 80% ethanol (low molecular weight solutes) then boiling water (polysaccharides), leaving a residual fraction (structural polysaccharides, proteins and nucleic acids). More sophisticated separation techniques must be used to quantify the amount of radioactivity within individual biomolecules, for example, column chromatography (pp. 348–9) and autoradiography (p. 407), or 2D thin layer chromatography (p. 347) and autoradiography. High specific activity $\text{H}^{14}\text{CO}_3^-$ and short incubation times of a few seconds are required to study the early stages in carbon photoassimilation.

Using chlorophyll fluorescence measurements – these can give insights into the stress responses of plants, e.g. the extent of photoinhibition under field conditions – further details are provided by Maxwell and Johnson (2000).

The use of the stable carbon isotopes ^{12}C and ^{13}C in photosynthetic studies is covered in detail within Chapter 73, including example calculations of the relative abundance of each stable isotope, and $\delta^{13}\text{C}$.

Measuring chlorophyll fluorescence

This can be used to provide a non-destructive indication of photosynthetic function. When light energy is absorbed by the light-harvesting apparatus of green plants, several reactions compete for the deactivation of excited chlorophyll molecules. Principally, there are three possible outcomes: (i) the light energy may be trapped and used to reduce photosystem II (PS II) and drive photochemical reactions (Fig. 64.1); (ii) the energy may be lost as heat; or (iii) the energy may be re-emitted as fluorescence, measured at wavelengths around 685 nm using a purpose-built chlorophyll fluorometer. Since these three processes are mutually exclusive, measurements of chlorophyll fluorescence can provide information about changes in photochemistry and heat dissipation, since the maximum potential fluorescence yield is ‘quenched’ (reduced) by (i) photochemical and (ii) non-photochemical processes. Most measurements are made using pulse-amplified modulated (PAM) fluorometers (Fig. 64.4), where the light source used to generate the chlorophyll fluorescence can be turned on and off at high frequency (pulse modulated), enabling the chlorophyll fluorescence signal to be separated from all other light, and therefore allowing measurements to be made in light-adapted leaves under ‘natural’ conditions.

To interpret fluorescence changes, the relative contribution of the two quenching processes must be established. The starting point is to consider what happens when a strong light is shone on a dark-adapted leaf: there is a rapid rise in chlorophyll fluorescence to a maximum value, as photosynthetic electron transport is rapidly saturated and no further electrons can be accepted by PS II. If the light remains on, the chlorophyll fluorescence falls progressively, due to photochemical processes (for example, activation of carbon-fixing enzymes) and non-photochemical processes (dissipation of chlorophyll energy as heat). By measuring changes in chlorophyll fluorescence, the relative contributions of these two processes can be quantified. At a practical level, this is achieved by first shining a single flash of high-intensity light (the saturating pulse) on to a leaf in the dark-adapted state and the resulting maximum fluorescence signal, F_m (see Fig. 64.5 for detail) is then used to determine the potential maximum quantum yield of PS II as:

$$\text{Maximum quantum yield PS II} = \frac{F_m - F_0}{F_m} \quad [64.2]$$

The leaf is then allowed to adapt to ambient light, and the saturation pulse is then reapplied: the reduction in maximum fluorescence (to F_m') is the result of non-photochemical quenching (NPQ), i.e. dissipation of energy as heat (Fig. 64.5). This can be determined from the relationship:

$$\text{NPQ} = \frac{F_m - F_m'}{F_m'} \quad [64.3]$$

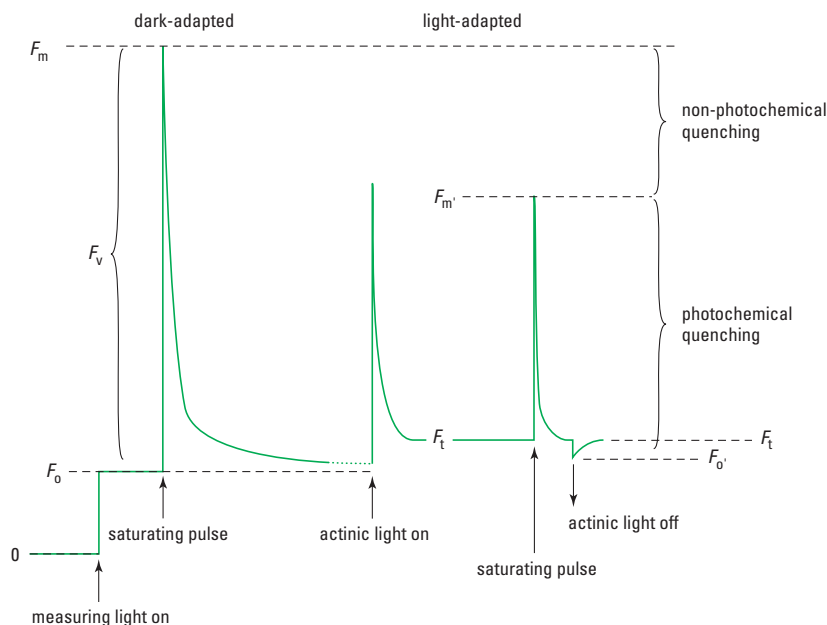
NPQ processes protect the protein components of the photosynthetic apparatus from oxidative damage due to excess light. The remaining



Fig. 64.4 A pulse-amplified modulated fluorometer (PAM), used to measure chlorophyll fluorescence (probe shown in left hand, attached to leaf, with analysis/recording unit on shoulder strap).

Courtesy of Heinz Walz GmbH, Effeltrich, Germany (<http://www.walz.com>).

Fig. 64.5 Representation of chlorophyll fluorescence measurement using the saturation pulse method (for details, see text).



Measuring photosynthetic quotients –

in studies where both O_2 and CO_2 are measured, the photosynthetic quotient (PQ) can be determined from the relationship: $PQ = O_2 \text{ evolved} \div CO_2 \text{ consumed}$. In the simplest case, where fixed carbon accumulates as carbohydrate, a value of 1 should be obtained. However, the PQ may vary, depending on the amount of carbon incorporated into fats, proteins, etc. and the utilisation of photosynthetic energy for other metabolic processes and growth, e.g. NO_3^- assimilation, carbon storage.

fraction is equivalent to photochemical quenching (qP), a measure of the proportion of PS II centres that are ‘open’, calculated from the relationship:

$$qP = \frac{F_{m'} - F_t}{F_{m'} - F_0'} \quad [64.4]$$

The quantum yield of PS II ($\Phi_{PS II}$), which is a measure of the efficiency of PS II photochemistry, can be determined as:

$$\Phi_{PS II} = \frac{F_{m'} - F_t}{F_{m'}} \quad [64.5]$$

The light and dark reactions of photosynthesis are coordinated, since electron transport generates the ATP and NADPH required for CO_2 fixation. For example, a reduction in the rate of CO_2 assimilation due to a metabolic inhibitor will be mirrored by a decrease in $\Phi_{PS II}$ and, in many cases, by an increase in NPQ as the light intensity is increased to levels in excess of those required for photosynthesis.

Studying photosynthetic electron transport

Robert Hill first showed that isolated chloroplasts can generate O_2 in the absence of CO_2 fixation, as long as they are provided with an artificial electron acceptor that intercepts electrons from the photosynthetic electron transport chain. The net result of this ‘Hill reaction’ is the photolysis of water and the reduction of the Hill acceptor (A), as follows:



The practical significance of the Hill reaction is that it allows the photochemical reactions of the electron transport system to be studied independently of the dark reactions of photosynthesis. Many of the Hill acceptors are redox dyes that show changes in absorbance as they are

Measuring the Hill reaction using DCPIP –

since the dye will revert to its oxidised (blue) form as soon as the chloroplasts are removed from the light, you must measure the absorbance as quickly as possible.

Table 64.1 Some inhibitors of photosynthetic electron transport

Inhibitor – target site
Hydroxylamine (NH ₂ OH) – photolysis of H ₂ O
DCMU (3(3,4-dichlorophenyl)-1,1-dimethylurea) – electron transport from PS II to plastoquinone
DBMIB (2,5-dibromo-3-methyl-6-isopropyl- <i>p</i> -benzoquinone) – electron flow from plastoquinone to cytochrome <i>f</i>
Methyl viologen (Paraquat) – electron flow from PS I to NADP ⁺
Atrazine (2-chloro-4-(2-propylamino)-6-ethylamine-5-triazine) – electron flow to plastoquinone
HOQNO (2-heptyl-4-hydroxyquinoline- <i>N</i> -oxide) – electron flow between quinones and cytochromes
DSPD (disalicylidinepropanediamine) – electron flow via ferredoxin

reduced, for example, ferricyanide (Fe(CN)₆³⁻), which accepts electrons from the PS I complex, or 2,6-dichlorophenolindophenol (DCPIP), which intercepts electrons from the electron transport chain between PS II and PS I (Fig. 64.1). The reduction of these artificial electron acceptors can be followed spectrophotometrically, allowing the Hill reaction to be quantified. Alternatively, an O₂ electrode can be used to follow O₂ evolution from PS II in the presence of various artificial electron acceptors. Redox mediators can be used to investigate the following aspects of photosynthetic electron flow:

- **the activity of PS II and PS I operating in series** can be studied in whole chloroplasts using a suitable terminal acceptor, for example, ferricyanide
- **the activity of PS II can be studied in whole chloroplasts** using DCPIP (membrane-permeable), or in fragmented chloroplasts using ferricyanide
- **the activity of PS I can be measured if PS II is blocked by the herbicide diuron (DCMU)** and if electron flow is maintained by the addition of an artificial electron donor, for example, ascorbate plus DCPIP. Note that this cannot be measured by O₂ evolution, since PS II is inoperative – methyl viologen can be used as an electron acceptor from PS I and is rapidly reoxidised, consuming O₂ in a reaction which can be measured using an O₂ electrode (as O₂ *uptake*, rather than O₂ *production*)
- **the sites of action of inhibitors (Table 64.1) can be determined** by measuring their effects on the various components of the electron transport system, measured using redox dyes or the methyl viologen/O₂ electrode system.

Quantifying respiration

At the cellular level, respiration can be considered as the oxidation of organic compounds coupled to the production of so-called high-energy intermediates, such as ATP. The overall process is often represented as:



the asterisks showing that O₂ is converted to water, and where *n* has a *maximum* value of 38. The process can be divided into three principal stages, namely (i) glycolysis, (ii) the tricarboxylic acid (TCA) cycle and

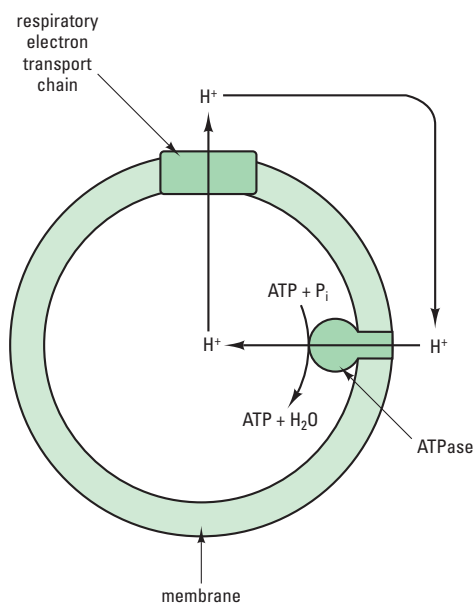


Fig. 64.6 Proton flow in mitochondrial oxidative phosphorylation: the creation of a trans-membrane H^+ gradient due to the respiratory electron transport chain drives ATP synthesis via a membrane-bound ATPase.

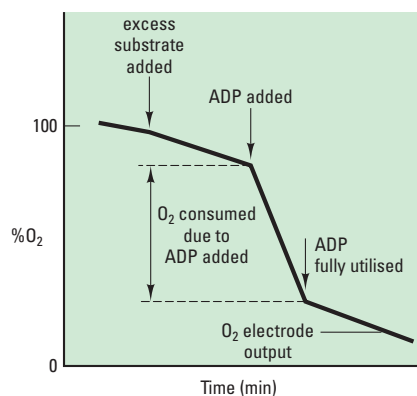


Fig. 64.7 Representative data for O_2 uptake by a suspension of mitochondria in response to additions of substrate and ADP.

Interpreting respiratory quotients – the complete oxidation of carbohydrates should give values close to 1.0, in agreement with eqn [64.8], while the oxidation of fats will give values close to 0.7 and protein oxidation will produce values of about 0.8.

(iii) oxidative phosphorylation. The glycolytic pathway operates in the cytosol and results in the partial breakdown of glucose (C_6) to two molecules of pyruvate (C_3) plus two molecules of NADH and two molecules of ATP. The mitochondrial TCA cycle involves the sequential dismantling of pyruvate to CO_2 (via an intermediate step involving decarboxylation to acetyl-coA), producing one molecule of $FADH_2$, four molecules of NADH and one of GTP for each pyruvate. The final stage occurs at the inner mitochondrial membrane – the movement of electrons and protons along the respiratory transport chain from reductant to O_2 as the terminal electron acceptor creates a transmembrane H^+ gradient that leads to the net synthesis of ATP, as summarised in Fig. 64.6. Thus, as in photosynthesis, respiration involves enzyme-catalysed interconversion of organic compounds plus membrane-bound electron transport reactions that are coupled to ATP synthesis.

The enzymic reactions of glycolysis and the TCA cycle can be studied using ^{14}C -labelled intermediates and pulse-chase experiments, using techniques similar to those used to investigate the dark reactions of photosynthesis (p. 481), or by purification and characterisation of the individual enzymes. Oxidative phosphorylation can be studied using techniques appropriate for electron transport reactions.

Measuring the respiratory activity of cells and tissues

The overall rate of cellular respiration is usually measured in terms of the amount of O_2 consumed by a known amount of material in a given time, for example, as $\mu\text{mol } O_2 \text{ mg protein}^{-1} \text{ min}^{-1}$.

The principal techniques include:

- **Manometry** – this traditional approach involves measuring either the pressure change (Warburg manometer) or volume change (Gilson manometer) as gases are produced or consumed during respiration. Manometry is relatively insensitive and may be subject to large measurement errors: the principal application is in studying the relationship between the amounts of O_2 consumed and CO_2 evolved in terms of the respiratory quotient (RQ) for a particular substrate, from the relationship:

$$RQ = \frac{CO_2 \text{ evolved}}{O_2 \text{ consumed}} \quad [64.8]$$

The calculations involved in determining CO_2 production or O_2 consumption are complex and are specific to individual instruments – manufacturer's guidelines should be followed carefully.

- **Oxygen electrode studies** – these have largely replaced manometry, as the apparatus is more versatile and less complex to set up and interpret, giving a continuous readout of O_2 status. Net O_2 evolution can be measured using whole cells, mitochondrial suspensions or submitochondrial preparations.

Using an oxygen electrode to study respiratory electron transport

Intact mitochondria suspended in an isotonic medium will show little respiratory activity unless supplied with (i) a suitable electron donor

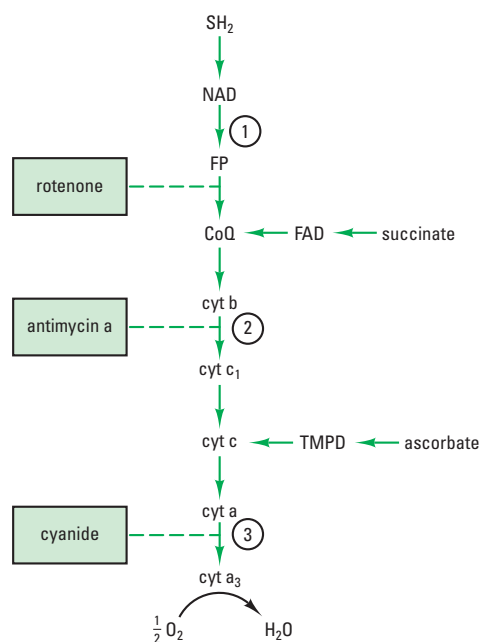


Fig. 64.8 The mitochondrial electron transport system, showing the sites of action of various inhibitors (dotted lines) and the three sites of ATP synthesis (numbered 1–3). S, substrate; FP, flavoprotein; CoQ, coenzyme Q; cyt, cytochrome; TMPD, tetramethylphenylenediamine.

or substrate (for example, NADH), (ii) ADP and (iii) P_i; this is termed respiratory control and the mitochondria are said to be tightly coupled, since there is a close link between electron transport, O₂ consumption and phosphorylation. The extent of this coupling can be determined using an O₂ electrode (p. 481) to measure O₂ consumption in the presence of substrate, P_i and ADP, with that in the absence of ADP (Fig. 64.7), as:

$$\text{respiratory control ratio} = \frac{\text{rate of O}_2 \text{ consumption with ADP}}{\text{rate of O}_2 \text{ consumption without ADP}} \quad [64.9]$$

Freshly prepared, tightly coupled mitochondria should have a respiratory control ratio of ≥ 4 .

To investigate the relationship between the number of ATP molecules produced per substrate molecule, substrate and P_i are supplied in excess and the O₂ uptake produced by a known amount of ADP is measured using an O₂ electrode (Fig. 64.8), allowing the P/O (\equiv ADP/O ratio for a particular substrate) to be calculated as:

$$\text{P/O ratio} = \frac{\mu\text{mol ADP added}}{2 \times \mu\text{mol O}_2 \text{ consumed}} \quad [64.10]$$

Note that the amount of O₂ is multiplied by 2, since O₂ is a diatomic molecule. The P/O ratio is determined by the site at which electrons are transferred to the respiratory chain (Fig. 64.9). Thus NADH should give a P/O ratio of 3, while succinate and FADH₂ should have values of 2. Artificial electron donors to cytochrome *c* have P/O ratios of 1, for example, ascorbate/tetramethylphenylenediamine (TMPD). However, measured P/O ratios are always less than these values, due to partial uncoupling and the action of ATPases.

The effects of inhibitors of respiratory electron transport on the rate of oxygen consumption of mitochondria can be used to determine their site of action (Fig. 64.8). Thus, an inhibitor of electron flow will prevent O₂ uptake while an artificial substrate that donates electrons at a location beyond the site of inhibition will restore O₂ uptake, as shown in Fig. 64.9.

‘Uncouplers’ act by increasing the permeability of membranes to protons, causing the dissipation of the transmembrane H⁺ gradient. Addition of an uncoupler increases electron flow along the respiratory chain in the absence of phosphorylation, i.e. there is loss of respiratory control. Uncouplers can reverse the effects of inhibitors such as oligomycin, whose target is the mitochondrial ATPase. Oligomycin prevents the return of H⁺ to the interior of the mitochondrion, thereby inhibiting respiratory electron transport and O₂ uptake – the addition of an uncoupler (for example, CCCP) reverses this inhibition.

KEY POINT When working with inhibitors, make sure you treat all of these as potentially toxic, observing appropriate safety precautions (Chapter 20), and that you know the procedure to be followed in case of accident or spillage.

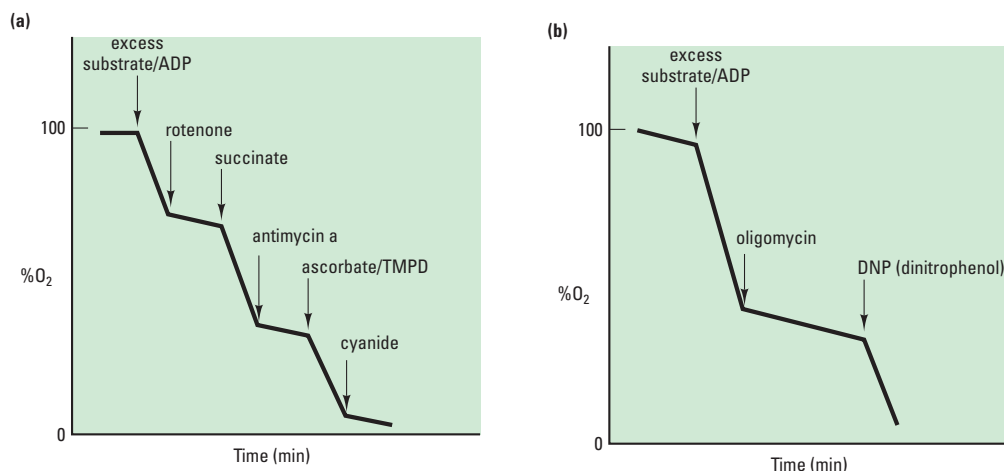


Fig. 64.9 Representative data for O₂ uptake by mitochondria in response to the addition of (a) electron transport inhibitors and substrates, or (b) inhibitors of ATP synthesis and uncouplers. Steeper slopes indicate faster rates of O₂ uptake.

Measuring the components of the proton-motive force in chloroplasts and mitochondria

According to chemiosmotic principles, the transmembrane electrochemical potential gradient of protons drives ATP synthesis in photophosphorylation and oxidative phosphorylation via a membrane-bound ATPase (Fig. 64.6). This gradient is often expressed as the proton-motive force, or PMF (Δp), expressed in mV as:

$$\Delta p = E_m - 59(\text{pH}_i - \text{pH}_o) \quad [64.11]$$

at 25 °C, where E_m is the transmembrane electrical potential (mV) and pH_i and pH_o are internal and external pH values. The individual components of the proton-motive force can be determined by a variety of methods:

- **transmembrane electrical potential**, by the distribution of a suitable radiolabelled lipophilic cation, for example, tetraphenylphosphonium (TPP^+), in accordance with the Nernst equation; by the measurement of K^+ or Rb^+ in the presence of the ionophore valinomycin; or by the quenching of a fluorescent dye (for example, cyanine or oxanol dyes)
- **transmembrane pH gradient** from the equilibrium distribution of a radiolabelled weak permeant acid, for example, 5,5-dimethyl-2,4-oxazolinedione (DMO); by quenching of a fluorescent pH probe, for example, 9-aminoacridine; or by ^{31}P -NMR.

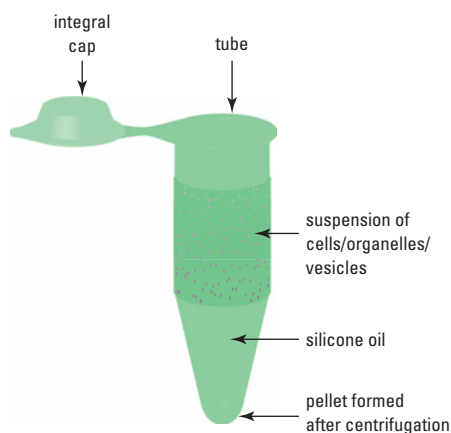


Fig. 64.10 Silicone oil microcentrifugation system: cells, organelles or membrane vesicles are pelleted below the silicone oil layer as a result of their higher density, while the less dense suspension medium remains above the silicone oil layer on centrifugation.

For radiolabelled probes, organelles or membrane vesicles can be separated from the bathing medium by silicone oil microcentrifugation (Fig. 64.10), with suitable correction for carry-over of external medium, for example, using a membrane-impermeant solute, labelled with a second radioisotope. Care is required in such double-labelled experiments, as carry-over can represent a significant component, leading to substantial measurement errors if uncorrected.

Typical measurements for Δp across energy-transducing membranes are 180–200 mV (inside-negative): in mitochondria, E_m is the principal component while ΔpH represents the largest component of Δp in thylakoid membranes.

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STUDY EXERCISES

(See also Study exercises 52.4 and 52.5 for calculations of respiratory oxygen consumption and photosynthetic oxygen evolution based on oxygen electrode measurements.)

64.1 Check your understanding of photosynthesis and respiration. Having read through this chapter, distinguish between each of the following pairs of terms:

- net photosynthesis and gross photosynthesis
- C₃ photosynthesis and C₄ photosynthesis
- the oxygenase and carboxylase functions of Rubisco
- coupling and uncoupling
- respiratory quotient and respiratory control ratio.

64.2 Calculate rates of photosynthetic carbon fixation from ¹⁴C data. A sample of the photosynthetic cyanobacterium *Spirulina platensis* containing 0.29 mg chlorophyll *a* (chl *a*) was incubated in the light for 3 min in 10 mL of an aqueous solution containing bicarbonate ions at a concentration of 12 mmol L⁻¹ and

labelled with ¹⁴C at a specific activity of 12 Bq pmol⁻¹ (≡ 0.72 d.p.m. nmol⁻¹). At the end of this period, the cyanobacterial cells were separated from the medium and assayed by liquid scintillation counting, giving a quench-corrected value of 2808 d.p.m. What is the photosynthetic rate, expressed in terms of μmol C fixed per mg chl *a* per minute? (Express your answer to three significant figures.)

64.3 Calculate the P/O ratio for a respiring mitochondrial suspension. An oxygen electrode chamber containing a mitochondrial suspension (0.5 mL), NADH (0.5 mL) and buffer (4 mL) showed a fall in oxygen concentration from 218 to 174 μmol L⁻¹ on addition of 0.05 mL of a 25 mmol L⁻¹ ADP solution. What was the P/O ratio over this period?

64.4 Interpret results from an experiment using DCPIP to study photosynthetic electron transport. The table below shows data for the change in A₆₀₀ of a chloroplast suspension incubated with the Hill acceptor DCPIP, plus different levels of the herbicide simazine.

(continued)

STUDY EXERCISES (Continued)

A_{600} measurements for a chloroplast suspension incubated with DCPIP and various levels of simazine

Time (s)	Control (no simazine)	Simazine at:		
		1.0 nmol L ⁻¹	10.0 nmol L ⁻¹	100.0 nmol L ⁻¹
0	0.462	0.461	0.464	0.462
30	0.372	0.394	0.41	0.436
60	0.320	0.345	0.371	0.413
90	0.284	0.315	0.343	0.397
120	0.253	0.284	0.317	0.385

- What is the reason for the rapid decrease in A_{600} in the control sample, with no added simazine?
- How do you account for the fact that increasing amounts of simazine lead to a reduction in the extent of change in A_{600} with time?

- Plot a graph of A_{600} against time to show the composite data from the table. Use this to determine the initial rate of change in A_{600} . Then, calculate the percentage inhibition at each simazine concentration. Finally, plot percentage inhibition against simazine concentration, to determine the concentration causing 50% inhibition (I_{50}); give your answer to three significant figures.

64.5 Calculate mitochondrial proton-motive force. A suspension of mitochondria in buffer at an external pH of 7.2 had a measured internal (matrix) pH of 8.6 and a measured transmembrane potential (inner membrane) of -85.6 mV. Calculate the proton-motive force across the inner membrane (express your answer to three significant figures). What is your interpretation of the calculated value?

Answers to these study exercises are available at go.pearson.com/uk/he/resources



Genetics and molecular biology

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65 Studying Mendelian and population genetics

Checking your understanding of key terms – before you read the rest of this chapter, carry out a self-assessment (p. 19) to make sure you know what the following terms and symbols mean, since they form part of the essential vocabulary of genetics (learn any that you are unable to define from memory):

- gene, allele; genome; locus
- genotype, phenotype
- chromosome, sex chromosome, autosome
- dominant, recessive, lethal
- haploid, diploid, gamete, zygote
- heterozygous, homozygous
- P, F1, F2.

Quoting chromosome numbers – remember that mitochondria and chloroplasts contain DNA molecules, forming part of an organism's genome, but these are not included when calculating the chromosome number.

Definitions

Meiosis – division of a diploid cell that results in haploid daughter cells carrying half the original number of chromosomes. Occurs during gamete (egg and sperm) formation.

Mitosis – division of a cell into two new cells, each with the same chromosome number. Occurs in somatic cells, e.g. during growth, development, repair, replacement.

Remember the difference between these two similar-sounding words – meiosis has an 'e' and 's' (=egg and sperm), whereas mitosis does not.

Gregor Mendel, an Austrian monk, made pioneering studies of the genetics of eukaryotic organisms in the middle of the nineteenth century. He made crosses between different forms of flowering plants. Through careful examination and numerical analysis of the observable characteristics (phenotype) of the parents and their progeny, Mendel was able to deduce much about their genetic characteristics (genotype). The principles derived from these experiments explain the basis of heredity, and hence underpin our understanding of sexual reproduction, bio-diversity and evolution.

Mendelian genetics is concerned primarily with the transmission of genetic information of eukaryotic organisms, as opposed to molecular genetics, which deals with the molecular details of the genome (see Chapter 67). The non-Mendelian genetics of bacteria and their viruses is covered in Chapter 66.

You are most likely to use the principles of Mendelian genetics in practical classes involving 'crosses' (mating) between two parents of different phenotype/genotype, while population genetics is most often studied in relation to stability or changes in alleles (different forms of a gene) within a population.

KEY POINT A common initial stumbling block in genetics is terminology. In many cases the definitions are interdependent, so your success in this subject depends on your grasp of all the definitions and underlying ideas explained below.

Understanding important terms and concepts in Mendelian genetics

Each character in the phenotype is controlled by the organism's genes (the basic physical and functional units of inheritance). Each gene (sequence of nucleotides in DNA) usually defines the amino acid sequence for a specific polypeptide, protein or RNA (a gene product) – often an enzyme or a structural protein. Proteins give rise to the phenotype through their activity in metabolism or their contribution to the organism's structure. The full complement of genes in an individual is known as its genome; see Chapter 67 for molecular aspects. Individual genes can exist in different allelic forms, each of which generally leads to a different form of the protein it codes for.

In eukaryotes, the genes are located in a particular sequence on chromosomes within the nucleus. The number of chromosomes per cell is characteristic for each organism (its chromosome number, n). For example, the chromosome number for man is 23. In cells of most 'higher' organisms, there are two of each of the chromosomes ($2n$). This is known as the diploid state. As a result of the process of meiosis, which precedes reproduction, special haploid cells are formed (gametes) that contain only one of each chromosome ($1n$). In sexual reproduction, haploid gametes from two individuals fuse to form a zygote, a diploid cell with a new genome, which gives rise to a new individual through the process of mitosis. Cell numbers are increased by this process, producing genetically identical cells.

Example In the garden pea, *Pisum sativum*, studied by Mendel, the yellow seed allele Y was found to be dominant over the green seed allele y. A cross of YY × yy genotypes would give rise to Yy in all of the F₁ generation, all of which would thus have the yellow seed phenotype. If the F₁ generation were interbred, this Yy × Yy cross would lead to progeny in the next, F₂, generation with the genotypes YY:Yy:yy in the expected ratio 1:2:1. The expected phenotype ratio would be 3:1 for yellow:green seed.

Organisms vary in the span of the diploid and haploid phases. In some ‘lower’ organisms, the haploid phase is the longer-lasting form; in most ‘higher’ organisms, the diploid phase is dominant. A life-cycle diagram can be used to show how the phases are organised and what life forms are involved in each case.

Since each diploid individual carries two of each chromosome, it has two copies of each gene in every cell. The number of alleles of each character present depends on whether the relevant genes are the same (homozygous) or whether they are different (heterozygous). Hence, while there may be many alleles for any given gene, an individual could have two at most, and might have only one if homozygous. This number is the result of the alleles present in the parental gametes that fused when the zygote was formed.

The basis of Mendel’s experiments and of many exercises in genetics are *crosses*, where two individuals showing particular phenotypes (parents, P generation) are mated and the phenotypes of the offspring (F₁ generation, from Latin *filius/filia*=brother/sister), are studied (see Box 65.1). If homozygous individuals carrying alternative alleles for a character are crossed, one of the alleles may be *dominant*, and all the F₁ generation will show that character in their phenotype. The character not evident is said to be *recessive*. Subsequent generations of crosses are numbered F₂, etc.

In describing crosses, geneticists denote each character with a letter of the alphabet, a capital letter being used for the dominant allele and lower case for the recessive. Taking for example a gene with dominant and recessive forms ‘A’ and ‘a’ respectively, there are three possibilities for each individual: it can be (a) homozygous recessive, aa; (b) homozygous dominant, AA; or (c) heterozygous, Aa.

One reason for dominance relates to the activity of an enzyme coded by the relevant gene; for example, Mendel’s yellow pea allele is dominant because the gene involved codes for an enzyme responsible for the breakdown of chlorophyll. In the homozygous recessive case, none of the functional enzyme will be present, chlorophyll breakdown cannot occur and the seeds remain green. In the homozygous dominant and heterozygous cases, functional enzyme will be present, leading to the breakdown of chlorophyll and thus yellow seeds. Not all alleles exhibit dominance in this form. In some cases, the heterozygous state results in a third phenotype (incomplete or partial dominance); in others, the heterozygous individual expresses both genotypes (codominance). Another possible situation is epistasis, in which one gene (the modifier gene) affects the expression of another gene that is independently inherited. Note also that many genes, such as those coding for human blood groups, have multiple alleles.

KEY POINT Genetics problems may well involve one of the ‘standard’ crosses shown in Box 65.1. Before tackling the problem, try to analyse the information provided to see if it fits one of these types of cross. Figure 65.1 is a flowchart detailing the steps you should take in answering genetics problems.

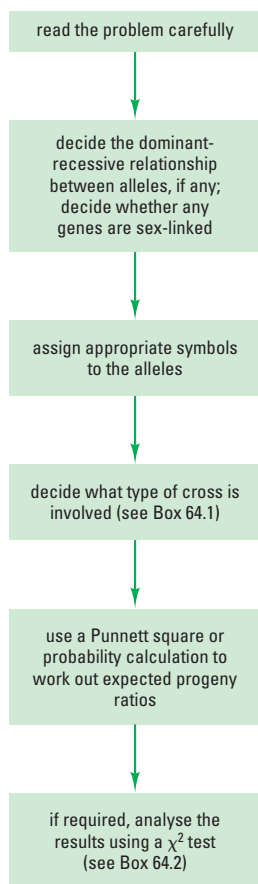


Fig. 65.1 Flowchart for tackling problems in Mendelian genetics.

Unless otherwise stated or obvious from the evidence, you should assume that the genes being considered in any given case are on separate chromosomes. This is important because it means that they will assort independently during meiosis. Thus, the fact that an allele of gene A is present in any individual will not influence the possibility of an allele

Box 65.1 How to interpret different types of genetic cross, and what you can learn from them

A. Monohybrid cross – the simplest form of cross, considering two alleles of a single gene.

Example: $AA \times aa$

If only the parental *phenotypes* are known, you cannot always deduce the parental genotypes from the phenotype ratio in the F_1 . An individual of dominant phenotype in the F_1 could arise from a homozygous dominant or heterozygous genotype. However, crossing the F_1 generation with themselves to give an F_2 generation may provide useful information from the phenotype ratios that are found.

B. Dihybrid cross – a cross involving two genes, each with two alleles.

Example: $AaBB \times AaBb$

As with a monohybrid cross, you cannot always deduce the parental genotype from the phenotype ratio in the F_1 generation alone.

C. Test cross – a cross of an unknown genotype with a homozygous recessive.

Example: $AABb \times aabb$

A test cross is one between an individual dominant for A and B with one recessive for both genes. The progeny will all be dominant for A, revealing the homozygous nature of the parent for this gene, but the progeny phenotypes will be split approximately 1 : 1 dominant to recessive for gene B, revealing the heterozygous nature of the parent for this gene. This type of cross reveals the unknown parental genotype in the proportions of phenotypes in the F_1 .

D. Sex-linked cross – a cross involving a gene carried on the X chromosome; this can be designated as dominant or recessive using appropriate superscripts (e.g. X^A and X^a).

Example: $X^A X^a \times X^A Y$

In sex-linked crosses you need to know the basis of sex determination in the species concerned – which of XX or

XY is male (for example, the former in birds, butterflies and moths, the latter in mammals and *Drosophila*). The expected ratios of phenotypes in the offspring will depend on this. Note that the recessive genotype will be expressed in the $X^a Y$ case.

E. Crosses with linked genes – genes are linked if they are on the same chromosome. This is revealed from a cross between individuals heterozygous and homozygous recessive for the relevant genes.

Example 1 (genes on separate chromosomes): expected offspring frequency from the cross $AaBb \times aabb$ is $AaBb$, $aabb$, $Aabb$ and $aaBb$ in the ratio 1:1:1:1.

Example 2 (linked genes): the frequencies of the last two combinations in example 1 might be skewed, depending on parental linkage.

Chromosome mapping uses the frequency of crossing-over of linked genes to estimate their distance apart on the chromosome on the basis that crossing-over is more likely the further apart are the genes. So-called 'map units' are calculated on the following basis:

$$\frac{\text{no. of recombinant progeny}}{\text{total no. of progeny}} \times 100 = \% \text{ crossing-over} \quad [65.1]$$

By convention, 1% crossing-over = 1 map unit (centimorgan, cM). The order of a number of genes can be worked out from their relative distances from each other. Thus, if genes A and B are 12 map units apart, while A and B are respectively 5 and 7 map units from C, the assumed order on the chromosome is ACB (Fig. 65.2).

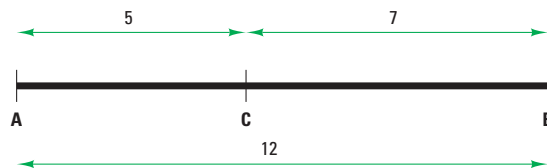


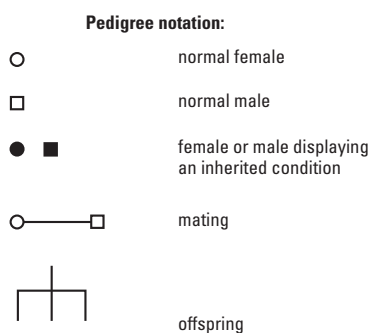
Fig. 65.2 Genetic map showing relative positions of genes A, B and C.

of gene B being present. This allows you to apply simple probability in predicting the genetic make-up of the offspring of any cross (see p. 498).

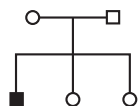
Where genes are present on the same chromosome, they are said to be *linked*, and thus it would be expected that they would not be able to assort independently during meiosis. However, although physically attached to each other on the same chromosome, they may become separated when

Denoting linked genes – these are often shown diagrammatically, with a double line indicating the chromosome pair. For example, the two possible linkages for the genotype AaBb would be shown as:

$$\begin{array}{c} \text{A} \quad \text{b} \\ \hline \text{a} \quad \text{B} \end{array} \quad \text{or} \quad \begin{array}{c} \text{A} \quad \text{B} \\ \hline \text{a} \quad \text{b} \end{array}$$



Example of simple family pedigree:



This diagram shows the offspring of a normal male and female. The two daughters are normal, but the son displays the inherited condition.

Fig. 65.3 Pedigree notation and family trees.

crossing-over occurs between homologous chromosomes at an early stage of meiosis. Exchange of genetic information between homologous chromosomes is called recombination and offspring that inherit a different combination of alleles at the two different loci (positions) on the same chromosome compared with their parents are known as recombinants.

Linkage can be detected from a cross between individuals heterozygous and homozygous recessive for the relevant genes, for example, AaBb × aabb. If the genes A and B are on different chromosomes, we expect the ratio of AaBb, aabb, Aabb and aaBb to be 1:1:1:1 in the F₁. However, if the dominant alleles of both genes occur on the same chromosome, the last two combinations will occur, but rarely. Just how rarely depends on how far apart they lie on the relevant chromosome – the further apart, the more likely it is that crossing-over will occur. This is the basis of chromosome mapping (see Box 65.1). Linkage can also be expressed in terms of the LOD (Log of the Odds) score, a statistical measure of how likely two genes are to be co-inherited. A LOD score of 3 or more is interpreted as meaning that the genes are located close to each other on the same chromosome.

Another feature you will come across is sex-linked genes. These occur on one of the X or Y chromosomes that control sex. Because one or other of the sexes – depending on the organism – is determined as XX and the other as XY (see Box 65.1), this means that recessive genes carried on the X chromosome may be expressed in XY individuals. You can sometimes spot sex-linked genes from differences in the frequencies of phenotypes in male and female offspring. Pedigree charts (Fig. 65.3) are codified family trees that are often used to show the inheritance and expression of sex-linked characteristics through various generations.

Studying Mendelian genetics using *Drosophila* as a model organism

The fruit fly *Drosophila melanogaster* is often used to demonstrate Mendelian genetics as it is relatively easy to culture, has a rapid life cycle (about 7 d from egg to adult at 28°C) and has only four pairs of chromosomes, including the sex chromosomes. Its genome has been sequenced and contains about 140×10^6 base pairs and nearly 16 000 genes. About 60% of these genes are also present in humans, including homologues for the genes involved in many human genetic diseases and other conditions, such as ageing, making it a useful model for human genetic research.

Drosophila are typically cultured in wide-necked plastic or glass bottles with a cotton wool or foam stopper, using a simple food medium containing hydrated, sterile ingredients – typically corn flour, glucose, sugar, and yeast powder, solidified with 2% w/v agar.

You may carry out crosses in your lab sessions, or you may be presented with the outcome of fruit fly crosses in the lab and asked to analyse the results (Box 65.2). This involves sorting the progeny into different types and testing theories with appropriate statistical tests. Flies will generally have been anaesthetised using carbon dioxide (for example, generated using a hydrated Alka-Seltzer tablet), ether or compounds like FlyNap or Lull-A-Fly, or you may be asked to do this yourself. Be prepared to work quickly before the effects of the anaesthetic wears off.

In some cases, you may be asked to carry out ‘virtual’ *Drosophila* crosses using programs such as *Drosophila* Genetics Lab (<https://www.newbyte.com/au/>).

Box 65.2 How to analyse the results of crosses involving *Drosophila*

To analyse the progeny of fruit fly crosses, you will first need to be familiar with the morphology of this insect, starting with the 'wild type' males and females from which mutants may differ (Fig. 65.4). Flies can be sexed on the basis of (a) overall size (female larger); (b) abdomen colour (male darker) and (c) presence of sex combs on forelegs (male only). As well as sexing flies, there are many anatomical features for which variants can be studied, including:

- **eye colour** (e.g. red eyes being dominant to white)
- **body colour** (e.g. light colour being dominant to ebony)
- **wing shape** (e.g. straight wings being dominant to dumpy wings).

Some differences in features may be tricky for novices to identify (e.g. vermilion vs red eye colour) – ask a demonstrator if unsure.

You can make counts of the progeny of crosses as follows:

1. **Mark a white card with areas for expected results of crosses**, e.g. 'white-eyed males'.

2. **Tip out anaesthetised flies onto the card for observation.** This may be placed on a cold plate to prolong anaesthetisation.
3. **Using a hand-lens or binocular microscope, examine each fly for the designated characteristics.** You may find it useful to have 'control' examples nearby with which to compare your flies.
4. **Move each fly to the appropriate area of your card with a small paint brush or dissection needle**, once you have decided its type.
5. **Keep a score of numbers in each card area**, e.g. using a tally chart (p. 548)
6. **Make statistical analysis of the observed results in relation to the expected results (Box 65.3).**

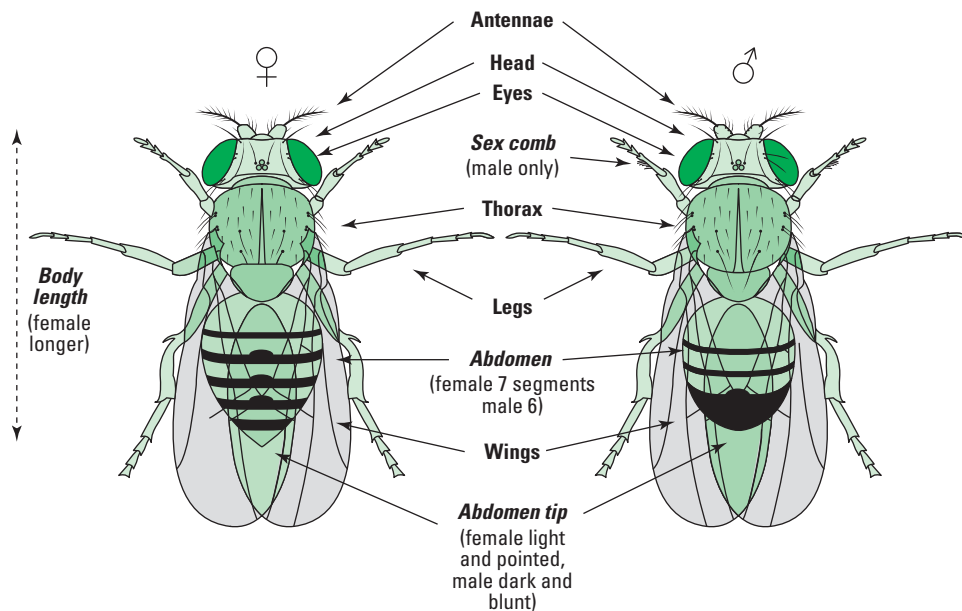


Fig. 65.4 Morphology of the fruit fly *Drosophila melanogaster*. Diagrams of female (left) and male (right) are shown, with labels indicating the main areas where easily-identified genetic mutations have been characterised. In terms of sexing flies (necessary for some crosses), the key identifiers are indicated by italics. Courtesy of András Somogyi.

Investigating Mendelian genetics in plants as model organisms – well-studied examples include the sweet pea *Lathyrus odoratus* (for example, flower colour) and the garden pea *Pisum sativum* (for example, smooth vs wrinkled; green vs yellow seed). Much has been learned from the study of thale cress, *Arabidopsis thaliana*, which has the advantage of a relatively low number of chromosomes, small size and a short generation time. All these species can be self-pollinated, which assists in the analysis of crosses.

Using probability calculations – these can be simpler and faster than Punnett squares when two or more genes are considered.

Carrying out genetic crosses – typically these are performed using organisms that have a large number of offspring, to even out random variation. Other useful attributes include short generation time, ease of maintenance, and wide range of mutations readily observed in the phenotype.

Analysing crosses

There are two basic ways of working out the results of crosses from known or assumed genotypes:

1. **The Punnett square method** provides a good visual indication of potential combinations of gametes for a given cross. Lay out your Punnett squares consistently as shown in Fig. 65.5. Then group together the like genotypes to work out the genotype ratio and proceed to work out the corresponding phenotype ratio if required.
2. **Probability calculations** are based on the fact that the chance of a number of independent events occurring is equal to the probabilities of each event occurring, multiplied together. Thus if the probability P of a child being a boy is 0.5 and the probability of the child of particular parents being blue-eyed is 0.5, then the probability of that couple having a blue-eyed son is $0.5 \times 0.5 = 0.25$, and that of having two blue-eyed boys is:

$$P = (0.5 \times 0.5) \times (0.5 \times 0.5) \times 0.0625$$

How do you decide whether the results of an experimental cross fit your expectation from theory as calculated above? This is not easy, because of the element of chance in fertilisation. Thus, while you might expect to see a 3:1 phenotype ratio of progeny for a given cross, in 500 offspring you might actually observe a ratio of 379:121, which is a ratio of just over 3.13:1. Can you conclude that this is significantly different from 3:1 in the context of random error? The answer to this problem comes from statistics. However, the answer is not certain, and your conclusion will be based on a balance of probabilities (see Box 65.3 and Table 65.1).

Investigating population genetics

Population genetics is largely concerned with the frequencies of alleles in a population and how these may change in time. The Hardy–Weinberg Principle states that the frequency of alleles f remains the same between generations, unless influenced by some outside factor(s). This notion was named after its first, independent protagonists, Godfrey H. Hardy and Wilhelm Weinberg. It is sometimes referred to as the Hardy–Weinberg equilibrium, theorem, law or principle.

		Male gametes	
		A	a
Female gametes	A	AA	Aa
	a	Aa	aa

Fig. 65.5 Layout for a simple Punnett square for the cross $Aa \times Aa$. The genotypic ratios for this cross are 1:2:1 for AA:Aa:aa, and the phenotypic ratio would be 3:1 for characteristic A to characteristic a. In this simple Punnett square, the allele frequencies are treated as equal ($f = 0.5$); if different from this, the probability of genotypes in each combination will be the relevant frequencies multiplied together.

Table 65.1 Values of χ^2 for which $P=0.05$. The value for $(n-1)$ degrees of freedom (d.f.) should be used, where n = the number of categories (=phenotypes) considered (normally fewer than 4 in genetics problems). If χ^2 is less than or equal to this value, accept the null hypothesis that the observed values arose by chance; if χ^2 is greater than this value, reject the null hypothesis and conclude that the difference between the observed and expected values is statistically significant.

Degrees of freedom	χ^2 value for which $P = 0.05$
1	3.84
2	5.99
3	7.82
4	9.49

Understanding the limitations of the Chi² test – note that the formula cited in Box 65.3 is valid only if expected numbers are greater than 5.

Box 65.3 How to carry out a Chi² (χ^2) test

This test allows you to assess the difference between observed (O) and expected (E) values and is extremely useful in biology. It is particularly valuable in determining whether progeny phenotype ratios fit your assumptions about their genotypes. The operation of the test is best illustrated by the use of an example. Assume that your null hypothesis (see pp. 602–3) is that the phenotypic ratio is 3:1 and you observe that in 500 offspring the phenotype ratio is 379:121, whereas the expected ratio is 375:125.

1. Start the test by calculating the test statistic χ^2 . The general formula for calculating χ^2 is:

$$\chi^2 = \sum \frac{(O-E)^2}{E} \quad [65.2]$$

In this example, this works out as:

$$\chi^2 = \frac{(379 - 375)^2}{375} + \frac{(121 - 125)^2}{125} = \frac{16}{375} + \frac{16}{125} = 0.171$$

2. Determine the probability associated with this value from χ^2 tables for $(n - 1)$ degrees of freedom (d.f.), where n = the number of categories = number of phenotypes considered. Here the d.f. value is $2 - 1 = 1$. Since the χ^2 value of 0.171 is lower than the tabulated value for 1 d.f. (3.84, Table 65.1), we therefore accept the null hypothesis and conclude that the difference between observed and expected results is not significant (since $P > 0.05$). Had χ^2 been ≥ 3.84 , then $P \leq 0.05$ and we would have rejected the null hypothesis and concluded that the difference was significant, i.e. that the progeny phenotype did not fit the expected ratio.

You can carry out χ^2 calculations using the CHITEST function in Microsoft Excel. The Help function within that program contains useful guidance.

To understand why the Hardy-Weinberg principle applies, consider two alleles H and h for a particular gene, which exist in the breeding population at frequencies p and q respectively. If the individuals carrying these alleles interbreed randomly, then the expected phenotype and allele ratios in the F_1 generation can be calculated simply as:

$$\begin{aligned} f(HH) &= p^2; \\ f(Hh) &= 2pq; \text{ and} \\ f(hh) &= q^2 \end{aligned}$$

If you wish to confirm this for yourself, lay out a Punnett square with appropriate frequencies for each allele. Now, by summation, the frequency of H in the $F_1 = p^2 + pq$ (a similar calculation can be made for allele h);

Example Cystic fibrosis occurs in 0.04% of Caucasian babies. If this condition results from a double recessive allele aa , then following the Hardy–Weinberg Principle and eqn [65.3], $q^2 = 0.0004$ (0.04% expressed as a fraction of 1) and so $q = \sqrt{0.0004} = 0.02$, or 2%. Since $p = (1 - q)$, $p = 0.98$, or 98%. The frequency of carriers of cystic fibrosis in the Caucasian population (people having the alleles Aa) is given by $2pq$. From the above, $2pq = 2(0.02 \times 0.98) = 0.0392$. Hence 3.92% of the Caucasian population are carriers (roughly one in 25).

and since in this example there are only two alleles, $p + q = 1$ and so $q = (1 - p)$ Substituting $(1 - p)$ for q , the frequency of H in the F_1 is thus:

$$p^2 - p(1 - p) = p^2 + p - p^2 = p \quad [65.3]$$

i.e. the frequency of the allele is unchanged between generations. A similar relationship exists for the other alleles.

The Hardy–Weinberg Principle holds so long as the following criteria are satisfied:

1. **random mating** – so that no factors influence each individual's choice of a mate
2. **large population size** – so that the laws of probability will apply
3. **no mutation** – so that no new alleles are formed
4. **no emigration, immigration or isolation** – so that there is no interchange of genes with other populations nor isolation of genes within the population
5. **no natural selection** – so that no alleles have a reproductive advantage over others.

Population geneticists use the Hardy–Weinberg Principle and eqn [65.3] to gain an idea of the rate of evolution. By ensuring that criteria 1–4 hold, if there are any changes in allele frequency between generations, then the rate of change of allele frequencies indicates the rate of evolutionary change (natural selection).

Sources for further study

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STUDY EXERCISES

65.1 Use a Punnett square to predict the outcome of a cross. Lay out a Punnett square for a cross between genotypes $RrOO \times RrOo$, where R is a semi-dominant gene for flower colour such that RR = red, Rr = pink and rr = white; and O is a dominant gene for corolla shape such that OO = closed corolla, Oo = closed corolla and oo = open corolla. From the Punnett square, derive both the genotypic and phenotypic ratios for the cross.

65.2 Use probability to predict the outcome of a cross. Two hazel-eyed parents are heterozygous for the eye-colour gene B. When expressed as bb , the individual is blue-eyed. Mum's hair is (genuine) blonde but Dad's is mousy-brown. In this case she is double recessive (mm) for the hair-colour gene M and he is heterozygous (Mm). What is the probability that they will have a blue-eyed, blonde daughter?

65.3 Work out a likely genetic scenario for a given set of results. Five tailless female mice were crossed with normal males (with tails). There were 31 normal mice and 28 tailless mice in the F_1 progeny. When pairs of tailless mice from the F_1 generation were crossed, their

(F_2) progeny were as follows: normal, 27; tailless, 55; dead on birth, but tailless, 30. In each case the ratio of males to females was roughly 50:50. Provide a logical explanation for these results.

65.4 Predict parental genotypes from the results of crosses involving sex-linked genes. A red-eye gene is known to be sex-linked in *Drosophila*; that is, the alleles R (red-eyed) or r (white-eyed) are carried on the X chromosome, while the Y chromosome does not carry these eye-colour alleles. In the fruit-fly, XX = female and XY = male. Predict the possible parental genotypes from the following F_1 progeny ratios:

- (a) 35 red-eyed female, 17 red-eyed male, 19 white-eyed male
- (b) 27 red-eyed male, 29 red-eyed female
- (c) 19 red-eyed female, 18 white-eyed female, 22 red-eyed male, 21 white-eyed male.

65.5 Carry out a χ^2 (Chi²) test. A geneticist expects the results of a test cross to be in the phenotype ratio 1:2:1. He observes 548 progeny from his cross in the ratio 125:303:120. What should he conclude?

Answers to these study exercises are available at go.pearson.com/uk/he/resources

66 Studying bacterial and phage genetics

Definitions

Copy number – the average number of copies of a particular cellular molecule.

Merozygote – a cell containing two copies of a part of its genome, i.e. a partial diploid (sometimes also termed a merodiploid).

Phage – a bacterial virus (bacteriophage).

Prophage – a bacterial virus genome integrated within the genome of a host bacterial cell.

Transposon – a section of DNA coding for its own movement from one genomic location to another and carrying other genes in addition to those coding for transposition.

In eukaryotic organisms, genetic reassortment usually involves the fusion of two haploid gametes to form a zygote and a new generation (p. 491). In many bacteria, the chromosome is a covalently closed circular DNA molecule, carrying genes for essential metabolic functions and structural components. As a consequence, bacteria can be regarded as haploid organisms. Plasmids are additional ‘mini-chromosomes’, typically coding for non-essential features, for example antibiotic resistance, heavy metal tolerance. They are often present at a higher copy number than the chromosome and may also carry genes within mobile transposable elements (transposons). A single bacterium may contain more than one type of plasmid (though not if they are closely related plasmids, i.e. from the same incompatibility group). Plasmids can be introduced into a bacterial cell by conjugation (p. 504) or by transformation (p. 502). Bacteria can be ‘cured’ of their plasmids by chemical treatment, for example using acridine dyes that interfere with replication, or by growth under particular conditions, for example at high temperatures, where plasmid replication may be unable to keep up with cell division.

KEY POINT Bacterial genetics is very different from eukaryotic Mendelian genetics (Chapter 65), due to the nature of the bacterial genome, which typically consists of a single chromosome plus none, one or several types of plasmid and/or phage/prophage, depending on the particular strain of bacterium.

Using and interpreting standard nomenclature in bacterial genetics – different forms of three-letter abbreviations are used for:

- **phenotypic features** – non-italic text, with superscripts where appropriate, e.g. Lac^+ ;
- **genotypic features** – lower case italic or underlined text, with individual letters to denote individual genes, e.g. *lacZ*;
- **gene products** (polypeptides and proteins) – the non-italicised equivalent of the abbreviation for the gene and with a capitalised first letter, e.g. the LacZ protein, which is a β -galactosidase and is the product of the *lacZ* gene in Lac^+ cells.
- **transposon** – inactivated genes show the transposon after the individual gene, separated by two colons, e.g. *lacZ::Tn6*.

Several abbreviations may be combined, e.g. a single strain might have the phenotype $\text{Amp}^r \text{Lac}^+ \text{Trp}^-$.

A phage may replicate inside a bacterial cell or, in selected instances (temperate phages, p. 507), may exist within the cell in a non-replicating (latent) state, termed a prophage. As such, phages represent additional genetic elements that may be present within a bacterial cell, forming an important component of several aspects of bacterial genetics at the practical level.

The principal characteristics of experimental bacterial genetics (‘crosses’) are:

- the processes are completely distinct from sexual reproduction in eukaryotes
- the processes are *directional*, from a donor cell (exogenote) to a recipient cell (endogenote)
- usually, only part of the donor cell’s genome is transferred
- in several instances, the recipient cell becomes a merozygote, with more than one copy of a gene, or genes. The merozygote may be a transient or a stable state, depending on circumstances
- recombination (synapsis and ‘crossing over’) may or may not be involved, depending on the process and strains involved
- transfer of genetic information may occur between donor and recipients of different species of bacteria; such horizontal gene transfer has been shown to be important in the spread of antibiotic resistance genes and the creation of multiple-resistant ‘superbugs’.

Definitions

Auxotrophs – mutants requiring an additional organic compound (e.g. an amino acid or growth factor) to grow in minimal medium.

Differential medium – usually a complex medium with additional compounds that distinguish between two types of bacteria, e.g. wild-type and mutant strains, often using pH indicator dyes or chromogenic/fluorogenic substrates.

Minimal medium – a chemically defined medium, containing only sufficient nutrients to meet the requirements of wild-type cells, i.e. inorganic salts plus a particular carbon source.

Prototrophs – wild-types (from which auxotrophs are derived) and all other strains capable of growth in minimal medium.

Visualising carbon source mutants – chromogenic and fluorogenic enzyme substrates (pp. 461–2) can be used to detect particular phenotypes; for example, *E. coli* mutant strains without a functional *lacZ* gene would give non-coloured colonies on a medium containing X-galactoside (p. 462), in contrast to wild-type strains, which would give blue-green colonies.

Taking care when distinguishing between carbon source and nutritional mutants – note that a *Lac*[−] mutant is unable to grow if provided with lactose as the sole carbon source, while a *Trp*[−] mutant is unable to grow unless it is provided with tryptophan as a specific nutrient.

KEY POINT Bacterial crosses are best described as gene *transfer* rather than gene *exchange*, since the latter term suggests reciprocal DNA movement.

Working with bacterial mutants

To study bacterial genetics, you need to make use of mutant strains, which have phenotypic characteristics that allow them to be distinguished from wild-type strains. The principal types of bacterial mutant include the following:

- **Morphological mutants**, with different structural characteristics from the wild-type, for example so-called ‘rough’ mutants of selected bacteria, such as *Streptococcus* and *Klebsiella*, are defective in their synthesis of capsular polysaccharides, giving small, dull colonies on agar-based media. This is in contrast to ‘smooth’ wild-type strains, where colonies are large and glistening owing to the hydrophilic polysaccharide capsule. At a practical level, it is relatively straightforward to work with such mutants, since wild-type and mutant bacteria will grow on the same medium, the mutants having a feature that visibly distinguishes them from wild-types. However, there are few examples in general use.
- **Resistant mutants**, which grow in the presence of an inhibitory substance such as an antibiotic, a toxic compound or a particular phage. As an example, the isolation of an ampicillin-resistant mutant (*Amp*^r phenotype) is made possible by including ampicillin in the growth medium, since the growth of sensitive wild-type cells (*Amp*^s phenotype) will be inhibited.
- **Carbon source mutants**, which are unable to use a particular substance as a source of carbon or energy, for example, *E. coli* mutants unable to use lactose (*Lac*[−] phenotype), in contrast to *Lac*⁺ wild-types. A *Lac*[−] mutant would be unable to grow on a minimal medium containing lactose as the principal carbon source. In order to identify and characterise such mutants, a differential medium must be used, for example MacConkey agar (a complex medium containing lactose as a *supplementary* carbon source, plus a pH indicator dye – colonies of *Lac*⁺ and *Lac*[−] strains are distinguished on the basis of size and pigmentation, p. 271).
- **Nutritional mutants**, which have an additional requirement for a particular nutrient, compared to the wild-type. For example, a strain auxotrophic for the amino acid tryptophan (*Trp*[−]) would grow only if the medium contained tryptophan (for example in a *minimal* medium plus tryptophan). Since it is not possible to devise a single medium that will allow an auxotroph to be distinguished from the corresponding prototrophic wild-type, the selection and identification of such mutants require a different approach, involving replica plating from (a) nutrient-rich medium onto (b) minimal medium and (c) minimal medium supplemented with the particular nutrient (Fig. 66.1).
- **Mutants created by transposon mutagenesis**, where a gene has been inactivated by the insertion of a transposon, for example *lacZ* inactivated by the insertion of Tn10 (p. 502).

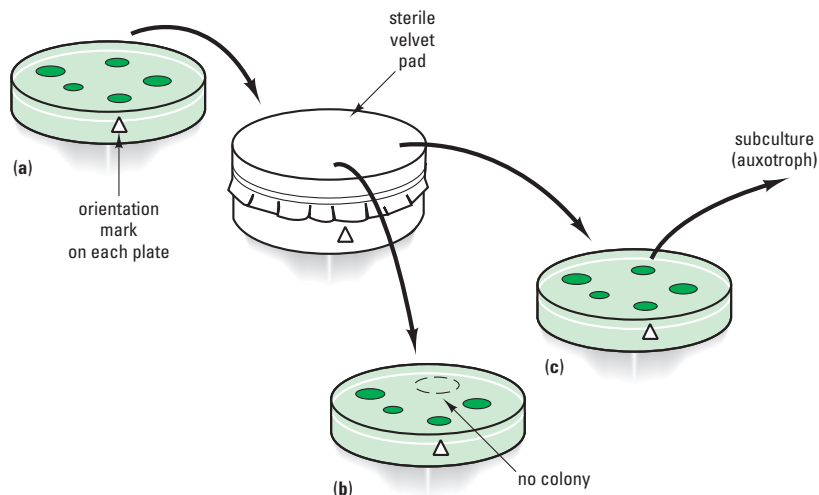


Fig. 66.1 Identification of auxotrophs using replica plating: a mixed suspension of wild-type and mutant cells is first cultured on the surface of a master plate containing nutrient-rich medium (a), then transferred to a sterile pad which is then used to inoculate minimal medium (b) and minimal medium plus a particular nutrient (c). Failure to grow on (b), coupled with growth on (c), implies auxotrophy for that particular nutrient and allows the auxotroph to be further subcultured and studied.

Locating mutants – there are several repositories of mutant bacterial strains; for example, the Genetic Stock Centre for *E. coli* at <http://cgsc.biology.yale.edu>.

- **Strains with different forms of a particular gene on the chromosome and also on a plasmid (partial diploids):** the genes carried on the plasmid can be shown as follows: *lacZ::Tn10/F' lacZ⁺* with the slash separating the transposon-inactivated chromosomal gene from the F' plasmid with its functional *lacZ* gene.
- **Conditional lethal mutants**, which have a defect that causes death under a specific set of circumstances (the 'restrictive condition'), for example in *E. coli* temperature-sensitive mutants that grow at 30 °C, but not 40 °C, often as a result of the temperature-dependent inactivation of mutant enzymes.

Understanding DNA transfer processes in bacteria

The principal bacterial gene transfer processes are described below.

Natural transformation

In contrast to genetic engineering techniques, where DNA uptake is induced under specific laboratory conditions (p. 532), natural transformation involves the release of DNA to the external medium (for example death and lysis of the donor cell) and its subsequent uptake and incorporation into the genome of the recipient cell. Transformation is restricted to a limited number of bacterial groups and only occurs if the recipient cells are in the correct physiological state, termed *competence*; this often occurs in the early exponential (log) growth phase (p. 242). Transformation is a relatively rare event, occurring at frequencies of ≤ 1 transformant per 10^3 cells. In a competent cell, DNA will be taken up and, if homologous (i.e. from another strain of the same species), may then be incorporated into the genome of the recipient cell by homologous recombination via a double 'crossover' (two recombination events, as shown in Fig. 66.2).

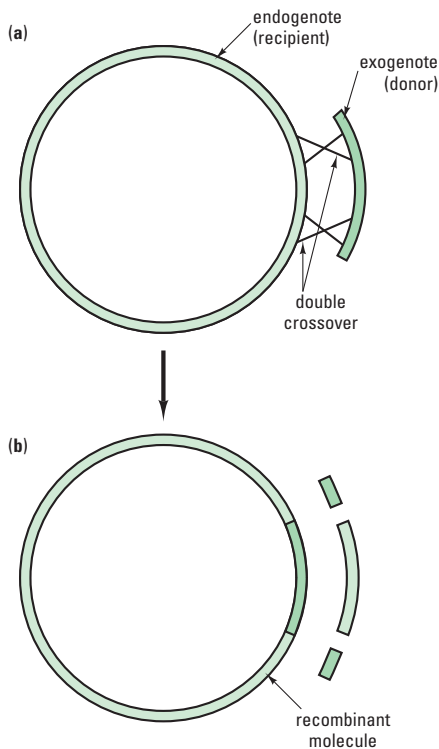


Fig. 66.2 Homologous recombination between donor DNA (from transformation, transduction or conjugation) and the recipient genome via a double crossover.

Performing co-transformation tests using auxotrophic recipients – controls must be set up without added donor DNA to determine the number of revertants (spontaneous mutants), giving the actual rate of transformation by subtraction.

Performing generalised transduction in *E. coli* – P1 phage is often used, since it gives a high proportion of transducing particles ($\approx 0.3\%$ of the total), with a large fragment size ($\approx 2.5\%$ of the *E. coli* genome), making it easier to locate individual transductants.

KEY POINT The traditional approach to genetic analysis in prokaryotes involves mapping the position of individual genes using information provided from ‘crosses’, based on gene transfer. Typically, the recipient cell will be a mutant and the donor DNA will carry wild-type genes, enabling the transfer of wild-type characteristics to be studied in the laboratory.

The principal application of transformation has been in mapping the position of genes in those bacteria showing natural competence (for example *Bacillus subtilis*). The experiments are often easiest to perform with auxotrophic recipients and prototrophic (wild-type) donor DNA, since transformants can be selectively grown on media lacking one or more individual nutrients. The frequency of co-transformation of two genes is a measure of how close together they are likely to be on the donor DNA strand – a high co-transformation frequency implies that they are close together on the chromosome, reaching a recipient cell on the same fragment of DNA.

KEY POINT While co-transformation frequencies are inversely related to map distances, they are not directly equivalent to the recombination frequencies used in mapping eukaryotic genomes (p. 289), because they are also influenced by the size distribution of the fragments of donor DNA and by the likelihood of homologous recombination.

Transformation mapping has several limitations, since it requires a fairly large number of complex replica plating experiments to produce a chromosomal map, and the relative position of genes that are very far apart cannot be determined directly – the ‘jigsaw’ requires a large number of available pieces, before the underlying structure can be seen. It is also insensitive for small map distances – two genes that are adjacent, or very close together, will give similar high co-transformation values.

Transduction

Here, the DNA exchange is mediated by a phage (p. 289), in one of two processes:

1. **Generalised transduction:** occasionally, a fragment of chromosomal or plasmid DNA within an infected bacterial cell may be packaged within the protein coat of a phage, in place of the phage genome. This fragment might be derived from any part of the host cell genome (exogenote). After release, on lysis of the donor cell, the transducing particle may introduce the DNA fragment into a new recipient cell. The introduced DNA may then be incorporated into the host genome by a double crossover, in a homologous recombination event similar to that shown in Fig. 66.2 for transformation. Generalised transduction can be used to establish gene order and for mapping purposes, using broadly similar principles to transformation, as only closely spaced genes will show co-transduction, with a co-transduction frequency that is inversely related to the distance between the two genes. Since a generalised transducing particle can carry a fairly small amount

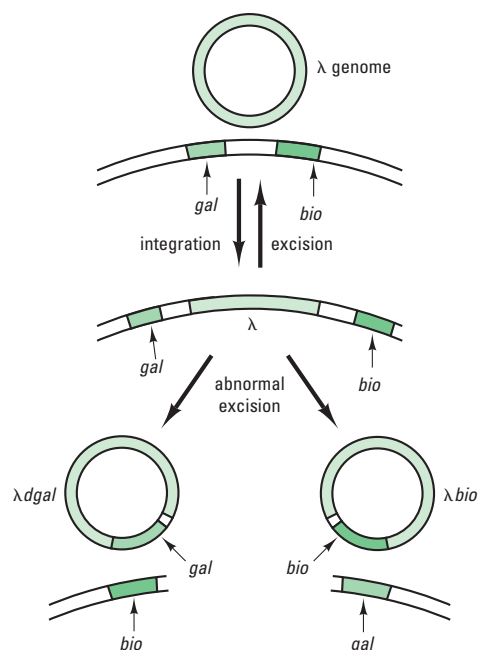


Fig. 66.3 Integration, excision and abnormal excision of λ phage, creating a modified phage genome (either $\lambda dgal$ or λbio), prior to specialised transduction.

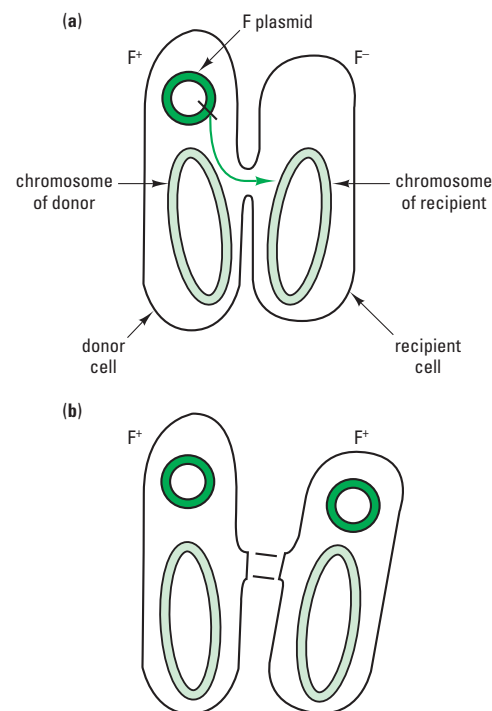


Fig. 66.4 Conjugation between F^+ donor and F^- recipient. A single strand of F plasmid DNA is cut and transferred in linear form to the recipient (a), followed by recircularisation and complementary strand synthesis, converting the recipient to F^+ (b).

of DNA, the relative frequency of co-transduction can be used to provide finer detail of gene order over shorter distances than for transformation.

2. **Specialised transduction:** this is mediated only by a temperate phage, for example λ phage, which integrates at a specific site on the chromosome by a single crossover event. It involves a restricted number of genes – typically, a pair of genes on either side of the integration site for a particular temperate phage (Fig. 66.3). Specialised transduction will only occur if, on entry into the lytic cycle (phage replication, p. 289), there is an incorrect (abnormal) excision of the prophage and a part of the bacterial genome is incorporated, giving a specialised transducing particle (a modified phage, carrying a bacterial gene). In the case of λ phage (Fig. 66.3), the transducing particle may carry either the biotin gene (λbio), or the galactose gene (λgal , also termed $\lambda dgal$, since the modified phage is defective owing to the loss of an essential part of the phage genome, and therefore is incapable of replicating without the aid of a co-infecting, non-defective ‘helper’ phage). On entry into the recipient cell, the modified phage may become latent, creating a partial diploid. The practical applications of specialised transduction are limited to those genes flanking the integration sites of temperate phages. The most common investigations are of complementation in merozygotes containing two copies of a particular gene. Where donor and recipients of the same mutant phenotype have mutations in different genes, the creation of a partial diploid can produce a wild-type phenotype.

The integration and excision of phages is regarded as an important means of gene transfer in natural environments, playing a key role in the evolution and pathogenesis of bacteria, as has been demonstrated by recent genome sequencing projects that have demonstrated the role of prophages in toxigenic *Vibrio cholerae*, *E. coli* and *Streptococcus pyogenes*.

Conjugation

Here, the transfer occurs as a result of cell-to-cell contact, with direct transmission of DNA from donor to recipient cell. In *E. coli*, the donor cell carries specific surface pili (protein microtubules), allowing a donor cell to attach to receptors on the surface of a recipient cell and bringing the paired cells into close contact (Fig. 66.4). In the simplest instance, the donor cell carries an additional plasmid, the F plasmid (originally termed ‘F factor’), that encodes the genes responsible for conjugation, including those for the protein subunits of the specialised pili. In conventional notation, the donor is termed F^+ and the recipient F^- . During conjugation between F^+ donor and F^- recipient, a single strand of the circular F plasmid is cut and transferred (in linear form) to the recipient cell, which becomes F^+ once the entire plasmid has been transferred and a complementary strand has been synthesised – this process takes a few minutes.

KEY POINT It is important to understand that the process of bacterial conjugation is completely different from sexual reproduction in eukaryotic organisms, since there are no gametes and no zygote or offspring is formed. Consequently, it is more appropriate to describe the participants as donor and recipient (of genetic information) rather than as male or female.

At a practical level, crosses involving F^+ donors give little useful information, apart from mapping the position of genes on the F plasmid. However, two other types of donor are more useful:

Example The *E. coli* strain HfrH has the F proplasmid integrated at a site close to genes for the synthesis of threonine, so these genes would be transferred early during HfrH \times F^- conjugation.

Example $F' lac$ is an F' plasmid that incorporates wild-type chromosomal DNA coding for lactose utilisation.

- Hfr strains**, where the F plasmid DNA has become integrated into the chromosome, as a proplasmid, in an analogous manner to a temperate phage such as λ (Fig. 66.3). Such strains show a *high frequency of recombination* (hence Hfr), since chromosomal genes are transferred to the recipient cell at a far greater frequency than in crosses using F^+ donors. The Hfr \times F^- cross is illustrated in Fig. 66.5: a part of the F proplasmid is first to be transferred, followed by chromosomal DNA and, finally, the remaining fragment of the F proplasmid. After transfer, donor chromosomal DNA can be integrated into the recipient's genome by homologous recombination (Fig. 66.2). The conjugating pair usually breaks apart before the process is complete and the recipient cell remains F^- , since only the leading fragment of the F plasmid reaches the recipient cell. A number of different Hfr strains of *E. coli* are available, with the F proplasmid inserted at different chromosomal locations.
- F' ('F prime') donors**, with a modified F plasmid incorporating one or more chromosomal genes, formed as a result of defective excision of the F proplasmid from the chromosome in a similar manner to λbio (Fig. 66.3). The F' plasmid will transfer its chromosomal genes at very high frequency to a recipient, which will also become F' once transfer of the F' plasmid DNA is complete. This process is sometimes referred to as F-duction (or, less appropriately, as 'sexduction').

Mapping with Hfr donors using interrupted conjugation

Mating an antibiotic-sensitive wild-type Hfr donor with an antibiotic-resistant mutant F^- recipient provides a simple means of detecting recombinants, since the donor cells will be unable to grow on a medium containing the antibiotic while unmodified recipient cells will have a different phenotype from the recombinant recipient cells. The method is even simpler for the most common crosses, using nutritional mutants, where a prototrophic Hfr donor is crossed with an auxotrophic F^- recipient and only the recombinants are able to grow on minimal medium with added antibiotic; this approach can be extended to multiple genes, using a multiple auxotroph F^- recipient. By carrying out a series of experiments where the conjugation process is terminated at different times (for example using vigorous mixing in a vortex mixer, p. 138), the time required to transfer a particular characteristic can be determined as the earliest time at which this interruption no longer prevents recombinants from appearing. Typical results from a cross involving several genetic markers are shown in Fig. 66.6(a): by plotting the result graphically and extrapolating the curves to their intersect with the x axis, the *time of entry* of a particular marker can be determined accurately.

The different times of entry of each characteristic reflect their relative positions on the chromosome, with genes near to the origin of transfer of the F proplasmid having short times of entry and those further along the chromosome having later times of entry. The times of entry can be used to locate the genes on a chromosome map (Fig. 66.6(b)), representing map

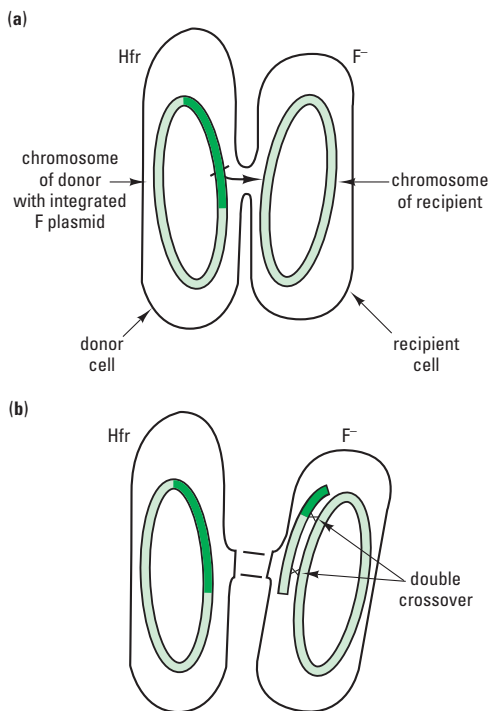


Fig. 66.5 Conjugation between Hfr donor and F^- recipient. The F proplasmid is cut and leads the chromosome (in single-stranded form) from the donor (a), followed by homologous recombination in the recipient (b).

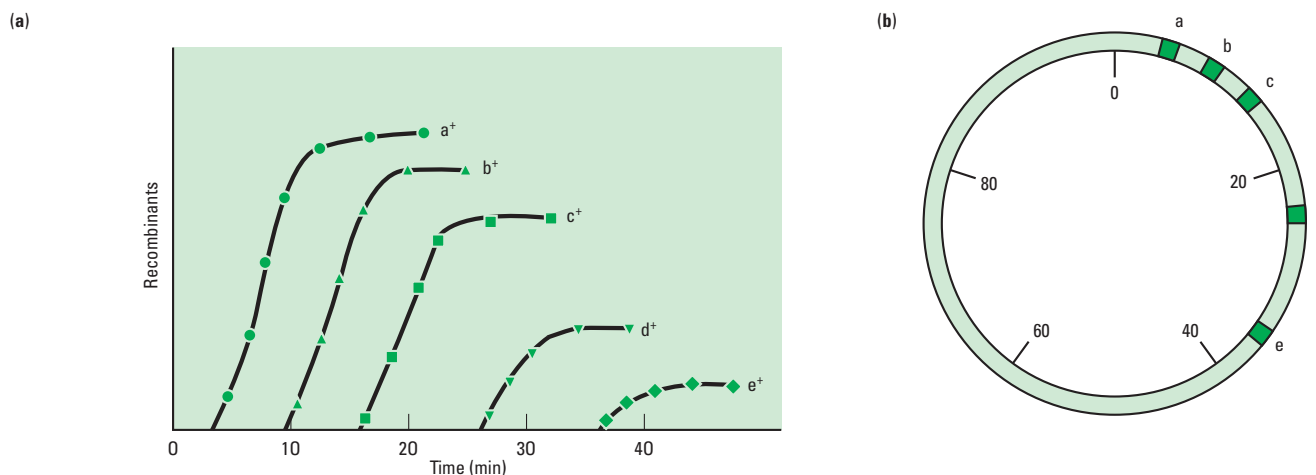


Fig. 66.6 Recombinants obtained from interrupted conjugation for a number of characters (a–e), plotted to show the time of entry of each character (a): the same data have been used to map the position of each character on the bacterial chromosome (b).

Mapping the genome using interrupted conjugation – while this technique works well for widely spaced markers, it is less useful than transformation and transduction for closely linked genes.

distances relative to the origin of transfer. This map differs from those produced from transformation and transduction analysis, since it is a transfer order map, rather than a linkage map. However, as transfer of the entire chromosome requires ≈ 100 min, it is difficult to map genes far from the origin of transfer, owing to the decreasing number of recombinants with increasing time (Fig. 66.6(a)): this is overcome by combining mapping data from several Hfr strains, each with the F plasmid integrated at a different position. This approach was originally used to demonstrate the circularity of the *E. coli* chromosome and to map the location of over 1500 individual genes.

Genetic analysis using F' plasmids

Since cells containing an F' plasmid are stable merozygotes, their principal use is in studying the behaviour of genes under diploid conditions; for example:

- **to determine whether a particular mutation (on the F' plasmid) is dominant or recessive over another (on the chromosome)**
- **to test whether a particular gene complements another:** if two mutations are in different genes, then they can complement each other in a stable merozygote, while two mutations in the same gene cannot complement each other in a stable merozygote
- **to examine whether a particular regulatory gene located on an F' plasmid influences the expression of one or more chromosomal genes;** i.e. whether it can operate in the *trans* position. The alternative test is to construct a merozygote with two genes in a chromosomal location (i.e. the *cis* position), for example, using specialised transduction (p. 504). This type of analysis has been used to investigate the molecular basis of gene expression and the function of regulatory regions of the chromosome.

Definitions

Cis (Latin 'here') – on the same DNA molecule.

Trans (Latin 'across') – on separate DNA molecules.

Performing phage crosses – large numbers of both types of phage must be used to ensure that every bacterial cell is infected with at least one of each type, i.e. there is a high multiplicity of infection.

Understanding conditional lethal mutants – an important group of phages are the so-called 'amber' mutants, with polypeptides containing a premature termination codon, UAG (p. 511); such mutant strains are propagated in 'amber suppressor' mutant strains of *E. coli*, which contain a mutant tRNA that recognises UAG as coding for an amino acid.

KEY POINT Note that, while many of the traditional procedures described in this chapter have proved to be useful in the early stages of bacterial genome analysis, the techniques and methods of molecular biology (Chapters 67–69) can be used to provide more detailed genetic information, without the need to obtain certain types of mutant, or carry out specific crosses between particular donors and recipients.

Understanding phage crosses

In practical classes, you may carry out experiments using phages, often using T-even phages of *E. coli* (for example T4 coliphage). The methods used to map phage genomes superficially resemble those used for eukaryotic organisms (Chapter 65) since the 'progeny' of two 'parental' phages with contrasting genotypes are analysed for recombinants and the frequency of recombination is used to measure the distance between linked genes.

KEY POINT A phage cross (sometimes informally described as a 'mating') does not involve meiosis, gamete production or zygote formation, but is a result of a *mixed infection*, where a single bacterial cell is infected with phages of the two original genotypes.

Following a single round of replication, the 'progeny' from this mixed infection are screened for the various possible genotypes, as plaques on a 'lawn' of a susceptible strain of *E. coli*, using conventional phage culture techniques (p. 289).

The phage genes most widely used in crosses are those affecting:

- **plaque morphology** – for example, mutants may form large plaques as opposed to small plaques, or plaques with a different margin, for example a light turbid halo as opposed to a dark halo
- **conditional lethality**, especially T4 phage mutants of the *rII* locus, unable to grow on *E. coli* K12(λ) in contrast to wild-type T4
- **host range** – for example T-even mutants may infect different strains of *E. coli* from the parental types.

Phage mapping

By crossing a mutant for plaque morphology with a mutant for host range, the relative frequency of recombinants (i.e. progeny with double mutant and wild-type phenotypes) can be expressed in terms of the proportion of the total number of plaques, as a recombination frequency (*R*), where:

$$R = \frac{\text{number of double mutants and wild-type plaques}}{\text{total number of plaques}} \quad [66.1]$$

This value is sometimes multiplied by 100 and expressed as a percentage, or as map units, equivalent to 'centimorgans' (1 cM = 1% crossing over) in conventional Mendelian crosses.

Learning from Benzer's work –

detailed fine structure mapping of the *rII* region of T4 coliphage established that recombination can occur within a single gene, and even between adjacent nucleotides. Before these experiments, a gene was regarded as an indivisible unit – nowadays we appreciate that the smallest unit in genetics is the base pair.

The pioneering work of Seymour Benzer established that the *rII* region of the T4 genome consisted of two distinct genes (*rIIA* and *rIIB*), each of which can give rise to the same mutant phenotype. T4 *rII* mutants are most often used for demonstrating *trans* complementation between these two genes and for demonstrating the principles of fine structure mapping of this region of the genome, since the conditional lethality of the *rII* phenotype simplifies the search for rare recombinant wild-type phages, as only these recombinants are able to form plaques on *E. coli* K12(λ).

Sources for further study

Birge, E.A. (2006) *Bacterial and Bacteriophage Genetics*, 5th edn. Springer, New York.

Casali, N. and Preston, A. (2003) *E. coli Plasmid Vectors: Methods and Applications*. Humana, New York.

Dale, J.W. and Park, S.F. (2010) *Molecular Genetics of Bacteria*, 5th edn. Wiley, Chichester.

Henkin, T.M. and Peters, J.E. (2020) *Snyder and Champness Molecular Genetics of Bacteria*, 5th edn. American Society for Microbiology Press, Washington, DC.

Snyder, L.A.S. (2020) *Bacterial Genetics and Genomics*. Garland, New York.

STUDY EXERCISES

66.1 Test your understanding of bacterial and phage genetics. Distinguish between each of the following pairs of terms:

- (a) zygote and merozygote;
- (b) transformation and transposition;
- (c) generalised and specialised transduction;
- (d) prototroph and auxotroph;
- (e) Hfr and F' strains.

66.2 Interpret the codes commonly used to describe bacterial mutant strains. Describe the *E. coli* phenotypes represented by the following:

- (a) Ampr Lac⁺ Trp⁻
- (b) His⁻ Lac⁻
- (c) *glnA::Tn10*
- (d) *trpA::Tn 6/F' trpA⁺*
- (e) a Gal⁺ strain lysogenised with λ*dgal*.

66.3 Interpret results from an Hfr mapping experiment.

Three different Hfr strains were used to map the genome of a bacterium in a series of interrupted conjugation experiments. The strains transferred genes to the F' recipient cell in the following orders:

Strain (a): X W C Z A

Strain (b): Y D F X W

Strain (c): Z A E B Y.

Assuming that all of these genes are present only once on a single circular chromosome, what is their order?

66.4 Construct a chromosomal map. The table below represents data for an interrupted conjugation study using an Hfr donor strain containing four 'marker' genes. These were distinguished by plating onto four different media, with the following major components:

(continued)

Medium A: glucose, histidine (trace amount), tryptophan (trace amount), ampicillin.

Medium B: glucose, histidine (trace amount).

Medium C: glucose, tryptophan (trace amount).

Medium D: lactose, histidine (trace amount), tryptophan (trace amount).

- (a) What is tested for by each of these four different media?
- (b) Based on the composition of these media and the data below, what is the phenotype of (i) the Hfr donor and (ii) the F^- recipient?
- (c) Given a chromosome map size of 60 min, where would each of these genes be located on the bacterial chromosome?

Colony numbers following interrupted mating, with plating on medium A, B, C or D.

Time (min)	Medium A	Medium B	Medium C	Medium D
0	0	0	0	0
10	0	32	0	0
20	0	287	38	0
30	34	339	182	0
40	156	341	226	28
50	179	338	229	89
60	180	340	227	95

Answers to these study exercises are available at go.pearson.com/uk/he/resources

67 Understanding molecular genetics – fundamental principles

Deoxyribonucleic acid (DNA) is the genetic material of all cellular organisms, while ribonucleic acid (RNA) is involved in protein synthesis. Consequently, the study of the structure and functions of these two biomolecules is a fundamental aspect of biology. The techniques described in this and the following two chapters are used widely in many areas of practical molecular biology, including the identification and characterization of genes, gene cloning and genetic engineering, medical genetics and genetic profiling. The main focus of this chapter is on methods and techniques for DNA, but those for RNA are often broadly similar. Structure and assay methods for both molecules are outlined in Chapter 60.

KEY POINT The sequence of the bases A, G, T and C carries the genetic information of the organism. A section of DNA that encodes the information for a single polypeptide, protein or functional RNA molecule is referred to as a gene, while the entire genetic information of an organism is termed the genome.

Abbreviating nucleic acid bases:

A = adenine
C = cytosine
G = guanine
T = thymine
U = uracil

Definition

Units of nucleic acid size (length) –
Kilobase pair (kbp or kb) = 10^3 base pairs.
Megabase pair (Mbp, or Mb) = 10^6 base pairs.

The amount of DNA in the genome is usually expressed in terms of base pairs (bp) or bases (b), rather than M_r , and its size depends on the complexity of the organism: for example, the human papillomavirus has a genome of 8×10^3 base pairs (8 kbp), that of *Escherichia coli* is 4×10^6 base pairs (4 Mbp), while the human haploid genome is very large, comprising in excess of 3×10^9 base pairs (3 Gbp). The human genome contains about 23 000 genes distributed across 23 pairs of chromosomes, representing about 1% of the total amount of genomic DNA (99% of human DNA is non-coding, but may be involved in other processes, including gene regulation). Organisms with smaller genomes have smaller amounts of non-coding DNA: some viral genomes have ‘overlapping’ genes, where the same base sequences carry information for more than one protein.

The size of each gene varies considerably: the largest ones may exceed 10 Mbp. Chromosomes represent the largest organisational units of DNA: in eukaryotes, they are usually linear molecules, complexed with protein and RNA, ranging in length from tens to hundreds of Mbp. The unit used to denote physical distance between genes (base pairs) differs from that used to describe genetic distance (centimorgan, cM), which is based on recombination frequency (p. 493). In humans, $1 \text{ cM} \approx 1 \text{ Mbp}$, though this relationship varies widely, depending on recombination frequency within particular regions of a chromosome.

Understanding gene transcription and translation

Before you investigate nucleic acid function, it is important to understand how genetic information is converted into biochemical outcomes. Each DNA template for the synthesis of RNA (transcription) begins at a promoter site upstream of the coding sequence (gene) and ends at a specific site at the end of the coding sequence (the terminator). The base sequence of this RNA is complementary to the ‘template strand’ and equivalent to the ‘coding strand’ of the DNA. In eukaryotic cells, transcription occurs

Table 67-1 The Genetic Code – combinations of nucleotide bases coding for individual amino acids.

1st Base	2nd Base				3rd Base
	U	C	A	G	
U	F	S	Y	C	U
	F	s	Y	C	C
	L	s	*	*	A
	L	S	*	W	G
C	L	P	H	R	U
	L	P	H	R	C
	L	P	Q	R	A
	L	P	Q	R	G
A	I	T	N	S	U
	I	T	N	S	C
	I	T	K	R	A
	M	T	K	R	G
G	V	A	D	G	U
	V	A	D	G	C
	V	A	E	G	A
	V	A	E	G	G

* = termination codons.

Standard abbreviations for the above amino acids are as given in Table 57.1.

Note that AUG (=M) is the initiation codon. The above codons are given for mRNA – the coding strand of DNA would have T in place of U, while the template strand would have complementary bases to those given above.

in the nucleus, where the newly synthesised RNA, or primary transcript, is also subject to processing, or ‘splicing’, in which non-coding regions within the gene (introns) are excised, joining the coding regions (exons) together into a continuous sequence. Further processing results in the addition of a polyadenyl ‘tail’ at the 3’ end and a 7-methylguanosine ‘cap’ at the 5’ end of what is now mature eukaryotic messenger RNA (mRNA). The mRNA then migrates from the nucleus to the cytoplasm, where it acts as a template for protein synthesis (translation) at the ribosome: the translated portion of mRNA is read in coding units, termed codons, consisting of three consecutive bases. Each codon corresponds to a specific amino acid including a codon for the initiation of protein synthesis (Table 67.1). Individual amino acids are brought to the ribosome by specific transfer RNA (tRNA) molecules that recognise particular codons. Individual amino acids are incorporated into the growing polypeptide chain in the order dictated by the sequence of codons on mRNA until a termination codon is recognised, after which the polypeptide/protein is released from the ribosome.

KEY POINT An important characteristic of nucleic acids is their ability to hybridise: two single strands with complementary base pairs will hydrogen bond (anneal) to produce a duplex, as in conventional double-stranded (ds) DNA (Fig. 60.4). This duplex can be converted to single-stranded (ss)DNA (i.e. ‘melted’) by conditions that disrupt hydrogen bonding – for example, by raising the temperature or adding salt – and then reannealed by reversing this disruption – for example, by lowering the temperature or removing salt.

Working with DNA

Standard purification and isolation methods enable the separation of intact, undenatured DNA from other biomolecules (Chapter 60), and it is relatively stable in its pure form. Most of the problems of working with such a large biomolecule have been overcome by the following techniques:

Following good practice in molecular genetics – careful technique is an essential aspect of practical work, due to the small volumes of samples and reagents used, coupled with the need to avoid contamination. Good practice includes:

- accurately pipetting down to 1 µL, or less
- steadying your hand during sample loading (p. 146, Fig. 22.5)
- keeping enzyme solutions cold during use and frozen during storage
- using sterile plasticware
- using ultrapure water
- wearing disposable gloves, to avoid contamination.

- **Enzymic fragmentation** – for example, the use of restriction enzymes (see below) can cut mammalian genomes into millions of fragments in a precise and reproducible manner, while small viral genomes may give only a few fragments. Fragmentation enables particular sequences to be identified through subsequent processing, as described below.
- **Electrophoretic separation** – typically based on the basis of the size of nucleic acid fragments. Such separation enables a particular fragment or ‘target sequence’ to be isolated and further studied.
- **Detection of ‘target’ sequences** – using ‘probes’ that specifically hybridise with the target nucleic acid, enabling it to be located and identified.
- **Determining the base sequence of fragments** – used together with strategies for combining information from multiple overlapping fragments to give contiguous sequences (‘contigs’). In this way, the sequence of an entire genome of an organism can be constructed (Chapter 70).

Example Clustal Omega software can be used for multiple sequence alignment – it can be used online via the European Bioinformatics Institute website at: <https://www.ebi.ac.uk/Tools/msa/clustalo/>

Definition

Plasmid – a DNA molecule that can replicate independently of the chromosome. Typically, these are configured as small circular molecules in bacteria and single-celled eukaryotic microbes (Chapter 66). Plasmids can be isolated, manipulated and introduced into bacterial cells.

- **Amplification of target sequences using the polymerase chain reaction (PCR)** – enabling the production of many copies of a particular sequence, as detailed in Chapter 68.
- **Producing large quantities of a gene or gene product through cloning** – this can be achieved by inserting the target sequence into a vector that is then introduced into a suitable host cell for multiplication, resulting from host cell division and proliferation (Chapter 69). Typically, cells transformed with the gene for a particular protein will be able to produce the protein in substantial amounts, with applications in medicine, biotechnology and related areas.

Isolating and purifying DNA

Specific details of the steps involved in the isolation of DNA vary, depending on the source material (Chapter 60). The sequence shown in Box 67.1 outlines the principles stages in the isolation of plasmid DNA from bacterial cells, as used in genetic manipulation (Chapter 69).

Producing DNA fragments

DNA can be fragmented by mechanical shearing or ultrasonication producing a random array of fragment sizes. In contrast, reproducible cleavage can be achieved using type II restriction endonucleases (commonly termed ‘restriction enzymes’) which recognise and cleave at a

Box 67.1 How to isolate plasmid DNA from bacteria

Plasmid DNA is smaller and more stable than chromosomal DNA, making it easier to work with in undergraduate practical classes. The step-wise process is:

1. **Digest the bacterial cell walls:** incubation in a lysozyme solution will remove the peptidoglycan cell wall, creating protoplasts/sphaeroplasts (p. 301). Carried out under isotonic conditions this will stop the cells from bursting open and releasing chromosomal DNA. Note that Gram-negative bacteria such as *Escherichia coli* are relatively insensitive to lysozyme, requiring additional treatment to allow the enzyme to penetrate the outer membrane and reach the cell wall layer; this can be achieved using osmotic shock, or incubation with a chelating agent, e.g. ethylenediaminetetraacetic (EDTA). The latter will also inactivate any bacterial deoxyribonucleases (DNases) in the solution, preventing enzymic degradation of plasmid DNA during extraction.
2. **Lyse the bacterial cells using strong alkali and a detergent:** typically, NaOH and sodium dodecyl sulphate (SDS) are used to solubilise cellular membranes and partially denature the proteins. Then neutralise this solution, for example, using potassium acetate: this will cause any chromosomal DNA to aggregate as an insoluble mass, leaving plasmid DNA in solution.
3. **Remove other macromolecules:** digest RNA and proteins using ribonuclease and proteinase. Additional chemical purification steps can give further increases in purity, e.g. proteins can be removed by mixing the extract with water-saturated phenol (50% v/v), or a phenol/chloroform mixture. On centrifugation, the DNA remains in the upper aqueous layer, while the proteins partition into the lower organic layer. Repeated cycles of phenol/chloroform extraction can be used to minimise the carry-over of these macromolecules. Note that phenol is toxic and corrosive and chloroform is potentially carcinogenic (Table 20.1). Take appropriate safety precautions (e.g. wear gloves, use a fume hood where available). If required, additional purification can be obtained using isopycnic density gradient centrifugation in CsCl.
4. **Precipitate plasmid DNA:** typically, add two volumes of 95% v/v ethanol to one volume of your aqueous extract, followed by centrifugation, to recover the plasmid DNA in the pellet. Further rinsing with 70% v/v ethanol: water will enable you to remove any salt contamination from the previous stages. If required for subsequent procedures, the extracted DNA can then be redissolved in buffer solution and frozen for storage.

Definition

Palindromic sequence – a double-stranded nucleic acid sequence that reads the same on one strand when read 'left-to-right' as on the other strand when read 'right-to-left' (see Fig. 67.1 for an example).

Extracting nucleic acids – many laboratories now use small-scale chromatographic columns for routine extraction of nucleic acids (Box 60.1). These are available in kit form, e.g. QIAprep Spin Minprep columns (p. 349).

Using bacterial antiviral defences in molecular genetics – clustered, regularly interspaced short palindromic repeat (CRISPR) nucleic acid sequences are recognised and cleaved by CRISPR-associated (Cas) enzymes as part of prokaryotic countermeasures to prevent replication of bacteriophages. The CRISPR-Cas9 system is widely used as the first stage in changing the genome of an organism at a particular site, opening up a target DNA sequence to enable editing of genes within living cells by site-directed mutagenesis (pp. 533–4). This was the basis of the award of the Nobel Prize for Chemistry, 2020 to Emmanuelle Charpentier and Jennifer Doudna.

Assaying nucleic acids in solution – double-stranded DNA at $50 \mu\text{g mL}^{-1}$ has an A_{260} of 1, and the same absorbance is obtained for single-stranded DNA at $33 \mu\text{g mL}^{-1}$ and (single-stranded) RNA at $40 \mu\text{g mL}^{-1}$. These values can be used to convert the absorbance of a test solution to a concentration of nucleic acid.

specific palindromic sequence of double-stranded DNA – usually four or six base pairs in length – known as the 'restriction site'. The term 'restriction' refers to the fact that these enzymes cleave foreign DNA within bacterial cells and thereby 'restrict' the multiplication of bacteriophages. Each restriction enzyme recognises a unique sequence and is given a code name derived from the name of the organism from which it is isolated – for example, *Hin* dIII was the third restriction enzyme to be obtained from *Haemophilus influenzae* strain Rd. Most restriction enzymes will cut each DNA strand at a slightly different position within the restriction site to produce short, single-stranded regions known as cohesive ends, or 'sticky ends', as shown for *Hin* dIII in Fig. 67.1. A few restriction enzymes (for example, *Sma* I) cleave DNA to give blunt-ended fragments, with no short, single-stranded regions.

Restriction enzymes have two important applications in molecular genetics:

- 1. Mapping.** A DNA molecule can be cleaved into *restriction fragments* whose number and size can be determined using agarose gel electrophoresis (see below). The position of individual sites can be used to create a *restriction enzyme map* for that particular molecule – for example, a plasmid (Fig. 69.1). Variation in restriction fragment lengths between individuals of the same species creates differences, termed *restriction fragment length polymorphisms* (RFLPs, pronounced 'riff-lips') – an early use of RFLP analysis was in forensic human DNA profiling, pioneered by Alec Jeffreys.
- 2. Genetic engineering.** Restriction fragments from different DNA molecules cut with the same enzyme can be annealed by the complementary base pairing of their 'sticky ends' prior to being joined together (ligated) using the enzyme DNA ligase, which forms covalent bonds between the annealed strands, creating a recombinant DNA molecule. When a target DNA sequence is cloned into a suitable plasmid vector and inserted into a host cell, easily detectable 'marker' genes (for example, antibiotic resistance) can be used to identify cells receiving the recombinant DNA (Chapter 69).

Separating nucleic acids using gel electrophoresis

Electrophoresis is the term used to describe the movement of ions in an applied electrical field (Chapters 50 and 51). The basic principles of this approach are common to DNA and RNA, since both carry a net negative charge due to the phosphate groups within their nucleotide subunits.

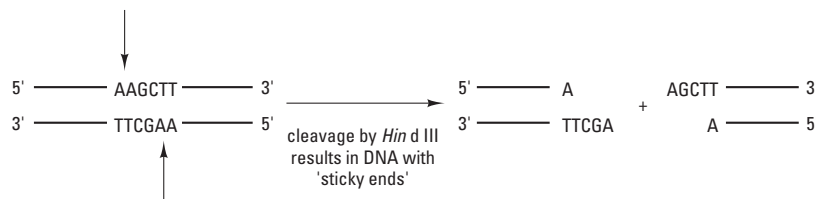


Fig. 67.1 Recognition base sequence and cleavage site for the restriction enzyme *Hin* dIII. This is the conventional representation of double-stranded DNA, showing the individual bases, where A is adenine, C cytosine, G guanine and T thymine. The cleavage site on each strand is shown by an arrow.

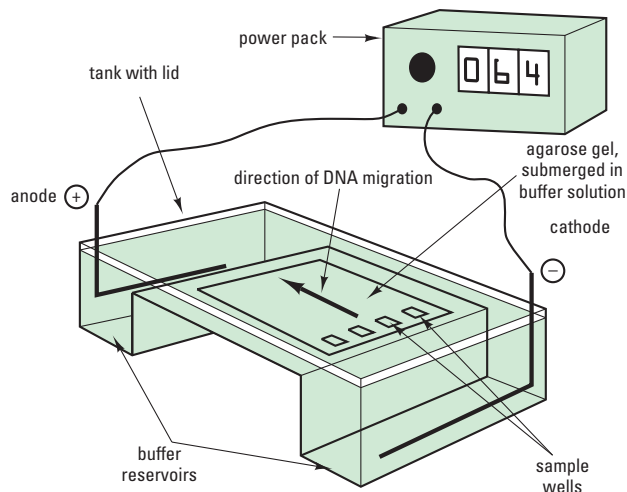


Fig. 67.2 Agarose gel electrophoresis of DNA.

Maximising recovery of DNA – these large molecules are easily damaged by mechanical forces, e.g. vigorous shaking or stirring during extraction. In addition, all glassware must be scrupulously cleaned and gloves must be worn, to prevent deoxyribonuclease contamination of solutions.

Working with small volumes in molecular biology – use a pipettor (autopipette) of appropriate size (e.g. P2 or P20 Gilson Pipetman) with a fine tip (p. 144). For very small volumes, pre-wet the tip before delivering the required volume.

Using DNA fragment size markers – for accurate determination of fragment size (length) your standards must have the same conformation as the DNA in your sample, i.e. linear DNA standards for linear (restriction) fragments and closed circular standards for plasmid DNA.

Electrophoretic separation of DNA

Negatively charged DNA molecules migrate through an agarose gel towards the anode at a rate that is dependent upon molecular size – smaller, compact DNA molecules can pass through the sieve-like agarose matrix more easily than large, extended fragments. Electrophoresis of plasmid DNA is usually carried out using a submerged agarose gel (Fig. 67.2). The amount of agarose is adjusted, depending on the size of the DNA molecules to be separated, for example 0.3% w/v agarose is used for large fragments (>20 000 bp) while up to 2% is used for smaller fragments. Very small fragments are best separated using a polyacrylamide gel (Table 67.2). Box 67.2 gives practical details.

The relative amount of DNA in your gel can be roughly estimated by visual comparison of stained bands with ‘marker’ bands of known quantity. A more accurate determination can be made using band densitometry, for example, using the GelDoc® system. Another quantitative approach is to extract the band from the gel and then measure the absorbance of the solution at 260 nm using a spectrophotometer, as explained in Chapter 60 (p. 448).

Table 67.2 Gel concentrations for the separation of DNA of various sizes.

Type of gel	% (w/v)	Range of resolution of DNA (bp)
Polyacrylamide	20.0	5–100
	15.0	20–150
	5.0	75–500
	3.5	100–1000
Agarose	2.0	100–5000
	1.2	200–8000
	0.8	400–20 000
	0.3	1000–70 000

Box 67.2 How to carry out agarose gel electrophoresis of DNA

- 1. Prepare the gel.** Typically, a small volume (10–20 mL) of buffer plus agarose is heated gently until the powder dissolves – take care not to overheat, or it will boil over. Gels are often cast with a small amount of a visualising dye, such as SYBR Safe.
- 2. Prepare your samples.** A small amount of sucrose or glycerol is usually added, to increase the density of the sample. You can also add a water-soluble anionic ‘tracking’ dye (e.g. bromophenol blue or xylene cyanol) to each sample, so that migration can be followed visually.
- 3. Load your samples on to the gel.** Slowly and carefully, add individual samples to the pre-formed wells using a pipettor (each sample should be retained within the well due to its higher density, compared with the buffer solution). The volume of sample you add to each well is small – typically less than 25 μL , so a very steady hand and careful dispensing are needed to pipette each sample accurately.
- 4. Load DNA markers on to the gel.** Typically, these standards of known size are added to the first and last wells of your gel: after electrophoresis, the relative positions of the DNA marker bands of known size can be used to prepare a calibration curve (Chapter 60), usually by plotting \log_{10} size (length) against distance travelled.
- 5. Carry out (‘run’) the electrophoresis.** DNA separation is usually carried out at 100–150 V for 30–60 min (see manufacturer’s instructions for specific details, according to which ‘power pack’ you are using); the gel should be run until the tracking dye has migrated across 80% of the gel.
- 6. Examine the gel and interpret the outcome.** If you have used a visualising dye within the agarose, then you simply transfer the gel to a UV transilluminator and look for bands of fluorescence corresponding to each DNA fragment.
- 7. Extract any DNA bands of interest.** If a particular band is required for further study, the piece of gel containing that band can be cut from the gel using either a clean scalpel or a specialised gel band cutter.

SAFETY NOTE Always wear suitable UV-filtering safety glasses or a UV-protective face shield when working with UV radiation, to protect your eyes. Use a digital camera to photograph your gel. Alternatively, a dedicated image capture system can be used, e.g. Bio-Rad GelDoc®.

Electrophoretic separation of RNA

Total cellular RNA or purified mRNA can be separated on the basis of size by electrophoretic separations similar to those described above for DNA fragments. However, under the conditions used to separate dsDNA, ssRNA molecules tend to develop secondary structure, leading to anomalous mobilities. To eliminate RNA secondary structure, samples can be pretreated by heating in dilute formamide or glyoxal, and subsequent electrophoresis is carried out using a ‘denaturing’ gel that includes buffers containing formaldehyde.

Pulsed field gel electrophoresis (PFGE)

If structural information is to be gained about large stretches of genomic DNA, then the order of the relatively short DNA segments (generated by the restriction enzymes described above) needs to be established. This is technically possible by techniques such as ‘chromosome walking’, but is time-consuming and potentially difficult, especially when dealing with large chromosomes such as those from yeast (a few Mbp) and humans (50–100 Mb), in contrast to the smaller genomes of bacteria and viruses. The technique of PFGE allows separation of DNA fragments of up to ≈ 12 Mb.

Very large DNA fragments (>100 kbp) can be generated from chromosomal DNA by the use of certain restriction enzymes that recognise

Definition

Chromosome walking – a method for analysing areas of interest in DNA, in which the end of a segment of DNA is used as a probe to locate other segments that overlap the first segment: long stretches of DNA can be analysed by subsequent use of probes made from the ends of successive overlapping segments.

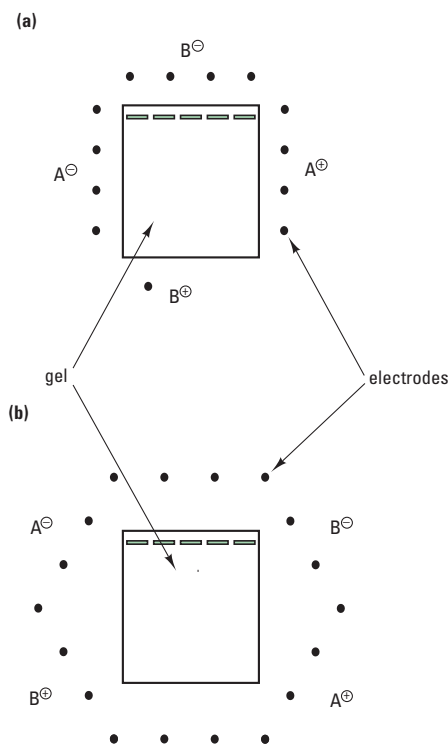


Fig. 67.3 The configuration of electrodes for conventional PFGE (a) and CHEF/PACE (b).

base sequences that are present at relatively low frequency, for example the enzyme *Not* I, which recognises a sequence of 8 bp rather than 4–6 bp. These enzymes are sometimes called ‘rare cutters’. Genomic DNA prepared in the normal way is not suitable for digestion by these enzymes, as shearing during extraction fragments the DNA. Therefore, genomic DNA for analysis by PFGE is prepared as follows:

- **cells are embedded in an agarose block**
- **the block is incubated in solutions containing detergent, RNase and proteinase K**, lysing the cells and hydrolysing RNA and proteins; the products of RNase and proteinase digestion diffuse away, leaving behind genomic DNA molecules exceeding several thousand kbp
- **the block is incubated in situ in a buffered solution containing an appropriate ‘rare cutter’**: restriction fragments are produced, of up to ≈ 800 kbp.

PFGE differs from conventional electrophoresis in that it uses two or more alternating electric fields. An explanation for the effectiveness of the technique is that large DNA fragments will be distorted by the voltage gradient, tending to elongate in the direction of the electric field and ‘snaking’ through pores in the gel. If the original electric field is removed, and a second is applied at an angle to the first, the DNA must re-orientate before it can migrate in the new direction. Larger (longer) DNA molecules will take more time to re-orientate than smaller molecules, resulting in size-dependent separations.

The original configuration of electrodes used in PFGE is shown in Fig. 67.3(a); this tends to produce ‘bent’ lanes that make lane-to-lane comparisons difficult. This can be overcome by using one of the many variants of the technique, one of which employs contour-clamped homogeneous electric fields (CHEF). Here, multiple electrodes are arranged in a hexagonal array around the gel (Fig. 67.3(b)) and these are used to generate homogeneous electric fields with reorientation angles of up to 120° . A further development of CHEF involves programmable, autonomously controlled electrodes (PACE), which allows virtually unlimited variation of field and pulsing configurations, and can fractionate DNA molecules from 100 bp to 6 Mbp.

Detecting specific nucleic acid sequences

Southern blotting

After separation by conventional agarose gel electrophoresis (Box 67.2), the fragments of DNA can be denatured and immobilised on a filter membrane using a technique named after its inventor, E.M. Southern. The principal stages in the procedure are shown in Box 67.3, while the main features of the conventional apparatus for Southern blotting are shown in Fig. 67.4. Modifications of the basic method use either a vacuum apparatus, an electric field (‘electroblotting’) or positive pressure to transfer the DNA fragments from the gel to the membrane, reducing the processing time by around 10-fold.

Northern blotting

This process is similar in principle to Southern blotting, but RNA is the molecule that is separated and probed.

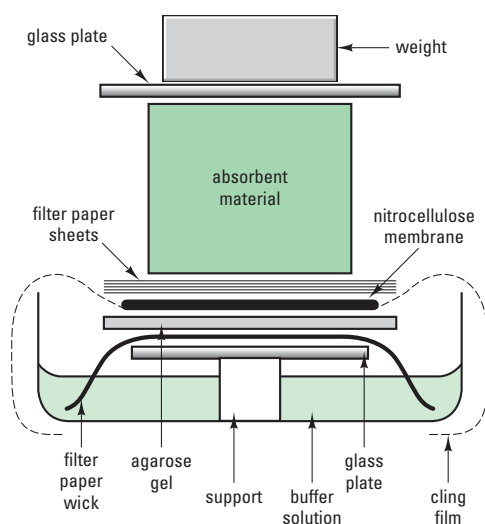


Fig. 67.4 Components of Southern blotting apparatus.

Box 67.3 How to carry out Southern Blotting to identify DNA

The text below outlines the process following electrophoresis (Box 67.2).

1. **Soak the agarose gel in acid (HCl)** – this leads to random cleavage of the DNA into smaller fragments.
2. **Soak the gel in alkali (NaOH)** – this denatures the double-stranded fragments (dsDNA), forming single-stranded fragments (ssDNA), then neutralise. This stage is necessary to allow hybridization with probe DNA after blotting.
3. **Place a nitrocellulose or nylon membrane on the gel, followed by several layers of absorbent paper.**

The DNA is transferred ('blotted') onto the filter as the buffer solution soaks into the paper by capillary action. Alternatively, this step can be replaced by suction transfer.

4. **Expose the filter to UV light or incubate ('bake') in a vacuum oven at 80°C.** This 'fixes' the DNA to the filter.
5. **Identify specific DNA sequences by incubation (6–24 h) with labelled ssDNA 'probes' (see below).** The probe and its complementary target sequence will hybridise, enabling the target sequence to be visualised – typically this will use a chromogenic detection system.

Understanding blotting terminology – following the style of 'Southern' blotting for DNA (named after its inventor) subsequent blotting methods have been described using points of the compass, with 'Northern' blotting for RNA, 'Western' blotting for proteins/antigens and, most recently, 'Eastern' blotting for post-translational modifications of proteins.

Definition

Probe – a labelled DNA or RNA sequence used to detect the presence of a complementary sequence (by molecular hybridisation) in a mixture of DNA or RNA fragments.

Example For a simple tripeptide containing methionine, aspartate and phenylalanine, the synthetic oligonucleotide probes would include combinations of the following codons:

1st codon (met): ATG
2nd codon (asp): GAT, GAC
3rd codon (phe): TTT, TTC

Dot blotting or slot blotting

Here, samples containing denatured DNA or RNA are applied directly to the nitrocellulose membrane (via small individual round or slot-like, templated holes) without prior digestion with restriction enzymes or electrophoretic separation. The 'blot' is then probed in a similar manner to that described for Southern blotting. This allows detection of a particular nucleic acid sequence in a sample. This approach has been used to detect specific pathogenic microbes in clinical samples and to identify particular genetic markers in inherited genetic disorders and forensic science.

Using nucleic acid probes

The probes used in blotting and hybridisation procedures can be obtained from a variety of sources, including:

- **cDNA** (complementary or copy DNA), which is produced from isolated mRNA using reverse transcriptase. This retroviral enzyme catalyses RNA-directed DNA synthesis, rather than the normal transcription of DNA to RNA. After the mRNA has been reverse transcribed, it is degraded by the addition of alkali or ribonuclease, leaving the ssDNA copy. This is then used as a template for a DNA polymerase, which directs the synthesis of the second complementary DNA strand to form dsDNA. This is denatured to ssDNA before use.
- **Oligonucleotide probes** (15–30 nucleotides) can be produced if the amino acid sequence of the gene product is known. Since the genetic code is degenerate, i.e. some amino acids are coded for by more than one codon (see Table 67.1), it may be necessary to synthesise a mixture of oligonucleotides to detect a particular DNA sequence: this mixture of oligonucleotides is termed a 'degenerate probe'.
- **Specific genomic DNA sequences**, where the gene has been characterised.
- **PCR-generated fragments** (Chapter 68).
- **Heterologous probes**, i.e. sequences for the same gene, or its equivalent, in another organism.

Using commercial kits – do not blindly follow the protocol given by the manufacturer without making sure you understand the principles of the method and the reasons for the procedure. This will help you to recognise when things go wrong and what you might be able to do about it.

Using labelled probes – an alternative to enzyme-linked and radiolabelled probes is the chemiluminescent system based on dioxigenin-labelled nucleotides.

Example Low stringency hybridisation can be useful when using a heterologous probe, i.e. from another species.

Example High stringency conditions can be used for detecting a single base change in a mutant gene using a dot-blot hybridisation procedure with a specific oligonucleotide probe.

Labelling of probes

Detection of very low concentrations of target DNA sequences requires probes that can be detected with high sensitivity. This is achieved using kits based on either enzyme-linked detection, or radiolabelling:

- **Enzyme-linked methods** involve incorporating a modified nucleotide precursor, such as biotinylated dTTP, into the DNA by nick-translation or random priming. When the probe hybridises with the target sequence, it can be detected by addition of an enzyme (e.g. horseradish peroxidase) coupled to streptavidin. The streptavidin binds specifically to the biotin attached to the probe and the addition of a suitable fluorogenic or chromogenic substrate for the enzyme allows the probe to be located.
- **Radiolabelled probes** can be made by several methods, including the nick-translation technique, to add an appropriate radionuclide, for example ^{35}S . This uses DNA polymerase I from *E. coli*, which has: (i) an exonuclease activity that ‘nicks’ dsDNA and removes a nucleotide, and (ii) a polymerase activity that can replace this with a labelled deoxyribonucleotide. After several cycles of nick-translation, the labelled DNA is denatured to ssDNA for use as probes. Newer approaches include random priming of single-stranded template DNA, followed by the synthesis of radiolabelled DNA fragments complementary to the template using a DNA polymerase and a radiolabelled deoxynucleoside triphosphate (dNTP). Probe hybridisation to a target sequence is detected by autoradiography. Oligonucleotide probes cannot be labelled using the above methods and require ‘end labelling’, where a kinase is used to replace the 5’ terminal phosphate group with a radiolabelled group.

Hybridisation of probes

The stability of the duplex formed between the probe and its target is directly proportional to the number and type of complementary base pairs that can be formed between them: stability increases with the amount of G + C, since these bases form three hydrogen bonds per base pair, rather than two between A+T. Duplex stability is also influenced by temperature, ionic strength and pH of the hybridisation buffer, and these can be varied to suit the stringency of hybridisation required:

- **‘Low stringency’ hybridisation**, involves duplex formation with less than perfect complementarity, and is promoted either by lowering the temperature, or by increasing ionic strength.
- **‘Stringent’ hybridisation conditions (high stringency)** usually involve high temperatures, or decreased ionic strength, and will sustain only perfectly matched duplexes.

Sequencing nucleic acids

By fragmenting target DNA with several restriction enzymes and then sequencing the overlapping fragments, it is possible to determine the nucleotide sequences of very large stretches of DNA, including entire genomes. Sequencing methods rely on polyacrylamide gel electrophoresis (p. 514). The Sanger, or chain termination, method is widely used in conventional DNA sequencing. This makes use of dideoxynucleoside

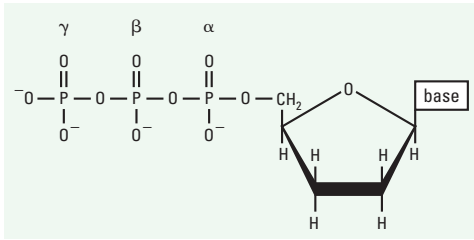


Fig. 67.5 A 2',3'-dideoxynucleoside triphosphate.

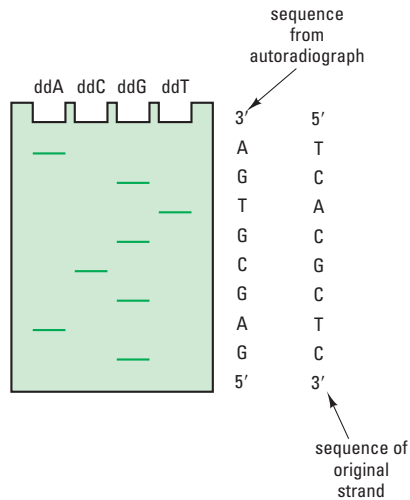


Fig. 67.6 Sanger sequencing gel, showing how the banding pattern is converted into a sequence of nucleotide bases.

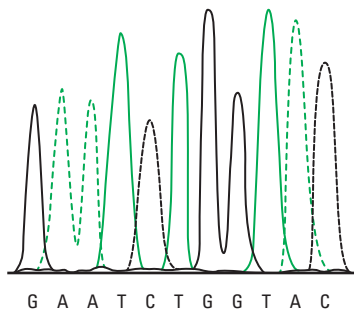


Fig. 67.7 DNA electropherogram.

Using PCR for automated sequencing

– with the four ddNTPs tagged using different fluorophores, the products can be run in the same lane and detected by laser excitation at the end of the gel, with the sequence of fluorophores giving the base sequence.

triphosphates (Fig. 67.5), which have no –OH group at either the C-2 or C-3 of ribose. A dideoxynucleoside triphosphate (ddNTP) can be added to a growing DNA chain, but since it lacks an –OH group at the C-3 position it cannot form a phosphodiester bond with the incoming dNTP of the growing chain. Therefore a dideoxynucleotide acts as a terminator at the site it occupies. Details of the Sanger method are given in Box 67.4 and Fig. 67.6.

Automated DNA sequencing

The development of automated DNA sequencing machines (so-called ‘DNA sequencers’) has enabled Sanger-based sequencing to be performed several orders of magnitude faster than with manual methods. Base-specificity is achieved by using primers labelled with fluorophores with different fluorescence characteristics in each of the four reaction tubes (Box 67.4). After the reactions are separately completed, the four sets of products can be pooled and fractionated by electrophoresis in a single lane. The fluorophore-labelled fragments are detected as they pass a scanning laser detector and the DNA sequence is determined by using both the specific wavelength emitted by the fragment (indicating the base) and by the migration time (indicating the fragment size, which corresponds to the base location in the DNA sequence). Such automated DNA sequencing tends to be performed by capillary electrophoresis (pp. 367–7) rather than polyacrylamide gel electrophoresis. An example electropherogram is shown in Fig. 67.7.

High-throughput sequencing

The quest for faster sequencing methods has led to a number of developments, collectively termed ‘high-throughput sequencing’, making it possible to sequence an entire genome in a fraction of the time required when conventional techniques are used and leading to rapid advances in genomics (Chapter 70). Several specific technologies have been developed for DNA sequencing, including:

- **Illumina sequencing** – a ‘short read’ parallel sequencing method that involves, first, fragmenting the target nucleic acid, then adding short ‘adapter’ sequences before analysing using a flow cell (for more details, see Illumina, 2021).
- **Oxford nanopore sequencing** – a ‘long read sequencing’ method that works by monitoring changes in electrical current as a single nucleic acid molecule passes through a protein nanopore – the electrical signal is then decoded to give the sequence of bases in the molecule, without any requirement for chemical modification or PCR amplification. For further details, see Nanopore, 2021)

Irrespective of the underlying chemistry, NGS methods work by simultaneously sequencing separate, clonally amplified DNA fragments (a ‘library’) in a massive array, with high throughput parallel sequencing operations numbered in the hundreds of thousands (for more details of the procedures involved in next-generation DNA sequencing, see Brown, 2015). A similar approach is taken in contemporary RNA sequencing, used to study the transcriptome (p. 536), rather than the genome (for more details, see Korpelainen *et al.*, 2014).

Box 67.4 How to carry out DNA sequencing using the chain termination (Sanger) method

DNA is first prepared as single-stranded fragments, and sequenced using a commercial kit with the following stages:

- 1. Strand synthesis.** Four separate tubes are prepared, each containing (i) target (template) DNA, (ii) a DNA polymerase, (iii) radiolabelled oligonucleotide primer of known sequence, (iv) dGTP, dATP, dCTP, dTTP in excess, plus, (v) one type of dideoxynucleotide in limited concentration, e.g. ddTTP to cause chain termination across sites where dTTP would normally be added. The reaction is terminated using a formamide solution, with incubation at 80 °C to produce ssDNA.
- 2. Fragment separation.** For example, using 4–6% denaturing acrylamide gel electrophoresis, with the products of the four reaction tubes in separate lanes, running at 35–40 W (up to 32 mA, 1.5 kV) for 2.5 h.

- 3. Autoradiography.** To locate individual bands. This requires vacuum-drying of the gel, then contact with an X-ray film for 24 h.

- 4. Reading the sequence.** Fig. 67.6 shows the process, with the sequence of the template strand being determined from the position of individual bands corresponding to the position of each nucleotide base.

Troubleshooting and other points to note: streaking can be due to damage to wells, air bubbles in wells/gel or contamination; faint/fuzzy gels can be due to insufficient template DNA or dNTPs and to poor annealing of primer and template; bands in the same position in more than one lane can be due to contamination, to multiple primer sites on the template, or to secondary DNA structure, giving 'ghost' banding.

DNA microarrays

Sometimes termed 'gene chips', DNA microarrays support the quantitative analysis of many different genes/sequences at the same time. This involves synthesising and fixing single-stranded probes for a large number (>1000) of different nucleotide sequences onto specific sites on a glass, quartz or silicon wafer. When single-stranded labelled nucleic acid is added, it will hybridise only with probes having a complementary sequence and these can be determined by the presence of an attached label, for example a fluorescent dye, detected using a scanning laser. One application involves the detection of labelled cDNA produced from the mRNA in an organism by using an 'expression' microchip, which contains probes for all of the expressed genes in an organism, enabling the relative expression of each gene to be determined from the fluorescence signal at each location on the microarray. Other applications include the use of single nucleotide polymorphism (SNP) microarrays to identify mutations and genetic variation, for example in screening for genetic diseases and in forensic DNA analysis. A commercial example is the Affymetrix GeneChip system (<https://www.affymetrix.com>).

Text references and sources for further study

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STUDY EXERCISES

67.1 Practise the calculations involved in working with nucleic acids. Imagine that you have been supplied with a freeze-dried sample of a single-stranded DNA probe 24 bases in length, which you reconstitute in 1 mL of buffer (reconstituted probe). You take 5 μL , add it to 495 μL of water and determine the A_{260} as 0.14.

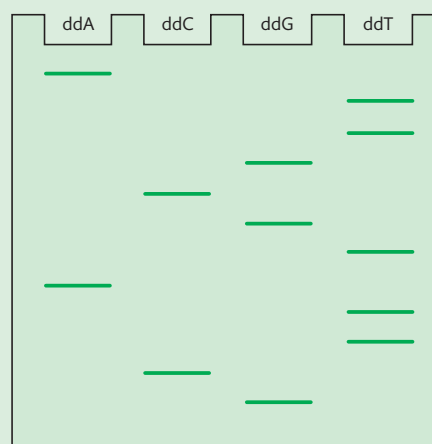
- What is the mass concentration of the probe in this solution, in $\mu\text{g mL}^{-1}$?
- Assuming the average M_r of a nucleotide is 325, what is the concentration of the probe in the solution, expressed in micromolar terms (i.e. as $\mu\text{mol L}^{-1}$)?
- The concentration of probe required in a blotting procedure is 10 pmol mL^{-1} and a total of 10 mL of this solution is required. You have been asked to prepare 5 mL of a stock solution of the DNA probe at $100\times$ the concentration required in the blotting procedure, so that 0.100 mL (100 μL) can be diluted to 10 mL for the final blotting procedure, while the rest will be stored at -20°C , for subsequent experiments. How would you prepare the $100\times$ stock solution?

Express all answers to three significant figures.

67.2 Select an electrophoretic technique for separating DNA fragments of different sizes. Which technique would you use to separate DNA fragments in the following ranges: (a) 1–70 kbp; (b) 75–500 bp; (c) 100–6 Mbp?

67.3 Test your understanding of the term 'stringency' in relation to nucleic acid probes. What does the term 'stringency' mean in the context of molecular biology? What factors cause increased stability between a nucleic acid probe and its target sequence? Give examples of when you might use 'low stringency' and 'high stringency' hybridisation.

67.4 Interpret the results of a Sanger sequencing gel. The figure represents a typical gel from a dideoxynucleotide sequencing procedure. Determine the sequence of the original DNA strand.



Answers to these study exercises are available at go.pearson.com/uk/he/resources

68 Using the polymerase chain reaction in molecular genetics

Example Applications of PCR include:

- diagnosis and screening of genetic diseases and cancer
- rapid detection of slowly growing microbes (e.g. mycobacteria) and viruses (e.g. HIV)
- HLA typing in transplantation
- analysis of DNA in archival material
- DNA profiling in forensic science
- preparation of nucleic acid probes (pp. 517–18)
- clone screening, mapping and subcloning (p. 524).

Understanding the terminology of PCR – cloned (amplified) DNA sequences produced using PCR techniques are often termed ‘amplicons’.

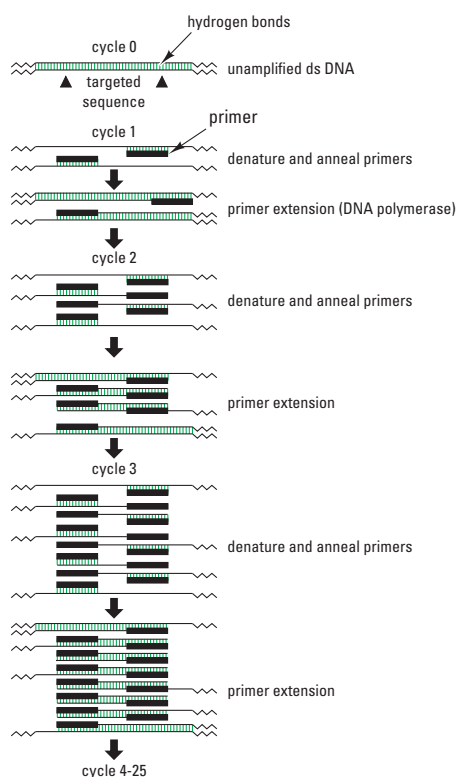


Fig. 68.1 The polymerase chain reaction (PCR).

The polymerase chain reaction (PCR) is a rapid and relatively inexpensive means of producing microgram amounts of DNA from minute quantities of template.

The technique uses *in vitro* enzyme-catalysed DNA synthesis to create millions of identical copies of DNA. If the base sequence of the regions adjacent to the DNA to be amplified is known, this enables synthetic oligonucleotide primers to be constructed that are complementary to these so-called ‘flanking regions’. Initiation of the PCR occurs when the synthetic primers are allowed to hybridise (anneal) to the component single strands of the target DNA, followed by enzymatic extension of the primers (from their 3' ends) using a thermostable DNA polymerase.

KEY POINT PCR offers an alternative approach to gene cloning (Chapter 69) for the production of many copies of an identical sequence of DNA. The starting material may be genomic DNA (e.g. from a single cell), RNA, DNA from archival specimens, cloned DNA or forensic samples.

A single PCR cycle consists of three distinct steps, (Fig. 68.1), carried out at different temperatures as follows:

1. **Denaturation of dsDNA** by heating to 94–98 °C, separating the individual strands of the target DNA.
2. **Annealing of the primers**, which occurs when the temperature is reduced to 37–65 °C.
3. **Extension of the primers** by a thermostable DNA polymerase (for example, *Taq* polymerase, isolated from *Thermus aquaticus*) at 72 °C; this step should last long enough to generate the PCR product: approximately 1 min of reaction time is required per kbp of sequence.

In the first cycle of the PCR, the product from one primer is extended beyond the region of complementarity of the other primer, so each newly synthesised strand can be used as a template for the primers in the second cycle (Fig. 68.1). Successive cycles will thus generate an exponentially increasing number of DNA fragments, the termini of which are bounded by the 5' ends of the primers (length of each fragment = length of primers + length of target sequence). Since the amount of DNA produced doubles in each cycle, the amount of DNA produced = 2^n , where n is the number of cycles. Up to 1 µg of amplified target DNA can be produced in 25–30 cycles from a single-copy sequence within 50 ng of genomic DNA, assuming close to 100% efficiency during the cycling process. After electrophoresis, the PCR product is normally present in sufficient quantity to be visualised directly, for example with ethidium bromide or SYBR Safe.

The temperature changes in PCR are normally achieved using a thermal cycler, which is simply a purpose-built incubator block that can be programmed to vary temperatures, incubation times and cycle numbers.

KEY POINT PCR is so sensitive that one of the main problems associated with the technique is contamination and amplification of 'foreign' DNA. Great care is required to avoid sample contamination during *in vitro* amplification (Box 68.1).

Designing PCR primers – online tools such as Primer Blast (at: <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) can provide primers specific to a particular template sequence.

Storing PCR primers – these often arrived in lyophilised form – resuspend in water and store in a freezer at -20°C or, preferably, at -80°C where they should be stable for several months.

Understanding codes for primers and probes – oligomeric nucleotides are often referred to by the number of bases, e.g. a 20-mer primer will contain 20 nucleotide bases.

Using dNTP solutions – make up stock solutions, divide into small volumes (50–100 μL of each dNTP) and store separately at -20°C .

Establishing the optimum concentration of Mg^{2+} – this can be determined by trial and error, and can be a useful starting point for any new PCR; try a range of different Mg^{2+} concentrations and select the one giving the strongest intensity of the target band following gel electrophoresis.

Understanding PCR components and conditions

Kits for carrying out PCR are readily available from commercial suppliers; they include a thermostable DNA polymerase, such as *Taq* polymerase, dNTPs, buffer, a detergent (for example, Triton), KCl , MgCl_2 or MgSO_4 , and primers. Protocols for PCR vary considerably for particular applications – a typical procedure is given in Box 68.1. However, if you are trying to develop your own PCR procedure in a lab project or assignment, the following information may be useful:

- **Primers need to be at least 18 nucleotides long** (primers longer than 18 nucleotides are likely to be unique, even in a large eukaryotic genome); both primers should have similar annealing temperatures, T_m , p. 449), with a minimal degree of self-complementarity (to avoid formation of secondary structures), and no complementarity to each other (so that primer dimers are not formed).
- **The final concentration of each primer should be $0.1\text{--}0.5\ \mu\text{mol L}^{-1}$** , which gives an excess of primers of about 10^7 with respect to the template, for example target genomic DNA at a concentration of 50 ng per 10 μL reaction mixture.
- **Annealing temperature should be determined according to T_m , the temperature at which 50% of the primers are annealed to their target sequence** (Fig. 60.7). For primers of < 20 bases, T_m can be roughly calculated in $^{\circ}\text{C}$ from the equation:

$$T_m = 4(G + C) + 2(A + T) \quad [68.1]$$

where G, C, A and T are the number of bases in the primer. Using this as a starting point, the optimum annealing temperature can be determined by trial and error. The T_m values of the two primers should be within 5°C of each other (and, therefore, be ideally identical in G + C content). The annealing temperature is then set 5°C below the lowest T_m of the primer pair.

- **dNTPs should be used at equal concentrations of $200\ \mu\text{mol L}^{-1}$ per dNTP**; this should provide the initial excess required for incorporation into DNA.
- **The last two bases at the 3' end (where elongation is initiated) should be either G or C** (3 hydrogen bonds, giving strong annealing), rather than either A or T (only 2 hydrogen bonds).
- **Mg^{2+} concentration is an important consideration**; Mg^{2+} is required as a co-factor for the thermostable DNA polymerase (Anon, 2021). Excess Mg^{2+} stabilises dsDNA and may prevent complete denaturation of product at each cycle; it also promotes spurious annealing of primers, leading to the formation of undesired products. However, very low Mg^{2+} concentration impairs polymerisation. Gelatin and Triton X-100 can be used to stabilise the DNA polymerase during thermal cycling.

Box 68.1 How to carry out the polymerase chain reaction (PCR)

The protocol given below is typical for a 'standard' PCR. Note that temperatures, incubation times and the number of cycles will vary with the particular application, as discussed in the text.

- 1. Make sure you have the required apparatus and reagents to hand**, including: (i) a thermal cycler; (ii) template thermostable DNA (≥ 50 ng/ μ L); (iii) stock solution of all dNTPs (5 mmol L⁻¹ for each dNTP); (iv) a DNA polymerase (at 5 U/ μ L); (v) primers at, e.g., 30 μ mol L⁻¹; (vi) stock buffer solution, e.g. containing 100 mmol L⁻¹ TRIS (pH 8.4), 500 mmol L⁻¹ KCl, 15 mmol L⁻¹ MgCl₂, 1% (w/v) gelatin, 1% (v/v) Triton X-100 (this stock is often termed '10 \times PCR buffer stock').
- 2. Prepare a reaction mixture**: for example, a mixture containing: 1.0 μ L target DNA; 2.5 μ L stock buffer solution; 1.0 μ L primer 1; 1.0 μ L primer 2; 1.0 μ L of each of the stock solutions of dNTPs, 0.1 μ L *Taq* polymerase; 15.4 μ L distilled deionised water, to give a total volume of 25.0 μ L.
- 3. Use appropriate positive and negative controls**: a positive control is a PCR template that is known to work under the conditions used in the laboratory, e.g. a plasmid, with appropriate primers, known to amplify at the annealing temperature to be used. A commonly used negative control is the PCR mixture minus the template DNA, though negative controls can be set up lacking any one of the reaction components.
- 4. Cycle in the thermal cycler**: for example, an initial period of 5 min at 94 °C, followed by 30 cycles of 94 °C for 1 min (denaturation), then 50 °C for 1 min (primer annealing; temperature depends upon G + C and A + T content, eqn [68.1] p. 523), then 72 °C for 1 min (chain extension).
- 5. Assess the effectiveness of the PCR**: for example, by gel electrophoresis and SYBR Safe staining.

Troubleshooting

- **If no PCR product is detected**, repeat the procedure, checking carefully that all components are added to the reaction mixture. If there is still no product, check that the annealing temperature is not too high, or the denaturing temperature is not too low.
- **If too many bands are present**, this may indicate that: (i) the primers may not be specific, (ii) the annealing

temperature is too low, or (iii) there is an excess of Mg²⁺, dNTPs, primers or enzyme.

- **If bands corresponding to primer-dimers are present**, this indicates that either: (i) the 3' ends of the primers show partial complementarity, (ii) the annealing temperature is not high enough, or (iii) the concentration of primers is too high.

Avoiding contamination in diagnostic PCR

The sensitivity of PCR is also the major drawback, since the technique is susceptible to contamination, particularly from DNA from the skin and hair of the operator, from previous PCR products, from airborne microbes and from positive controls. A number of routine precautions can be taken to avoid such contamination:

- **Use a laminar-flow cabinet** (p. 238) dedicated to PCR use and located in a separate lab from that used to store PCR products or prepare clones.
- **Keep separate supplies of pipettors, tips, microfuge tubes and reagents** – these should be exclusive to the PCR, with separate sets for sample preparation, reagents and product analysis.
- **Autoclave all buffers, distilled deionised water, pipette tips and tubes** prior to use.
- **Wear disposable gloves at all times and change them frequently**: protective coverings for the face and hair are also advisable.
- **Avoid contamination due to carry-over by including dUTP in the PCR mixture instead of dTTP**. Thus copies will contain U rather than T. Before the template denaturing step, treat the mixture with uracil-N-glycosylase (UNG, available commercially as AmpErase): this will destroy any strands containing U, i.e. any strands carried over from a previous reaction, or any contaminating material from another PCR. The target DNA will contain T, rather than U and will not be degraded by UNG. At the first heating step, the UNG will be denatured, so any newly synthesised U-containing copies will remain intact.
- **Use a strong UVC light** (p. 326) **inside the PCR workstation for 20–30 minutes before starting your work**, to degrade any contaminating DNA.

SAFETY NOTE – you must not expose your skin or eyes to any UVC source due to its mutagenic/carcinogenic properties.

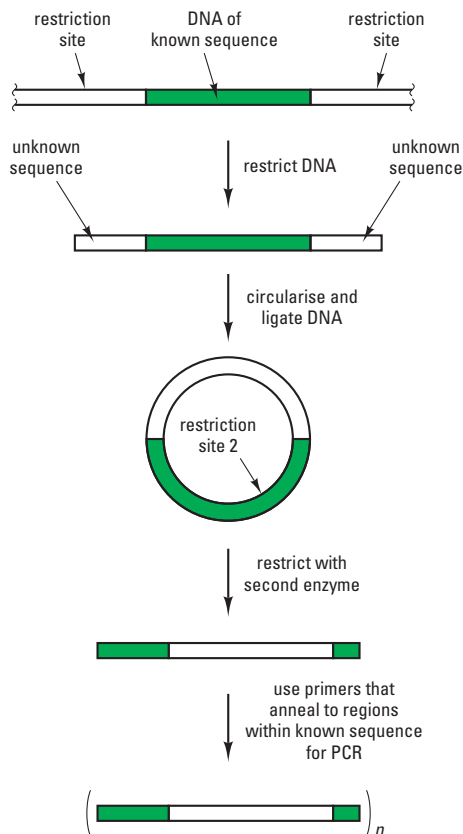


Fig. 68.2 Inverse PCR – basic principles.

Example Random amplification of polymorphic DNA (RAPD-PCR) can be used as a form of ‘molecular typing’ to identify a particular strain of an organism, e.g. in tracing the route of transmission of a pathogenic microbe.

- **Taq polymerase is a widely used thermostable DNA polymerase**, which extends primers at a rate of 2–4 kb per min at 72°C. It should be used at a concentration of $\geq 1 \text{ nmol L}^{-1}$ ($\geq 0.1 \text{ U per } 5 \mu\text{L}$ reaction mixture). A disadvantage of *Taq* polymerase is that it has a relatively high rate of misincorporation of bases (around one aberrant nucleotide per 100 000 nucleotides per cycle). Other polymerases are available (for example, *Pfu* polymerase from *Pyrococcus furiosus*, KOD polymerase from *Thermococcus kodakarensis* or genetically modified forms of *Taq* polymerase), which have lower misincorporation rates.

Using PCR variations

Nested PCR

This can be used when the target sequence is known, but the number of DNA copies is very small (for example, a single DNA molecule from a microbial genome), or if the sample is degraded (for example, a forensic sample). The process involves two consecutive ‘rounds’ of PCR. The first PCR uses so-called ‘external’ primers, and the second PCR uses two ‘internal’ (or ‘nested’) primers that anneal to sequences within the product of the first PCR. This increases the likelihood of amplification of the target sequences by selecting for it using different primers during each round. Thus, nested PCR also increases the specificity of the reaction, since a single set of primers used in isolation may give a reasonable yield but several bands, while the use of a second set of primers ensures that a unique sequence is amplified, for example in microbial diagnostics.

Inverse PCR

This is a useful technique for amplifying a DNA sequence flanking a region of known base sequence (Fig. 68.2), for example to provide material for characterising an unknown region of DNA. The DNA is cut with a restriction enzyme so that both the region of known sequence and the flanking regions are included. This restriction fragment is then circularised and cut with a second restriction enzyme with specificity for a region in the known sequence. The now linear DNA will have part of the known sequence at each terminus, and by using primers that anneal to these parts of the known sequence, the unknown region can be amplified by conventional PCR. The product can then be sequenced and characterised (Chapter 67).

Reverse transcription PCR (RT-PCR)

This technique is useful for detecting cell-specific gene expression (as evident by the presence of specific mRNA) when the amount of biological material is limited. Using either an oligo-dT primer to anneal to the 3′ polyadenyl ‘tail’ of the mRNA, or random hexamer primers, together with reverse transcriptase, cDNA is produced which is then amplified by PCR. RT-PCR is often a useful method of generating a probe, the identity of which can be confirmed by sequencing (pp. 518–20).

Amplification fragment length polymorphism (AFLP)

This term refers to several closely related techniques in which a single oligonucleotide primer of arbitrary sequence is used in a PCR reaction under conditions of low stringency, so that the primer is able to anneal to a large number of different sites within the target DNA. Some of the multiple amplification products will be polymorphic (for example, the presence or absence of a particular annealing site will result in presence or

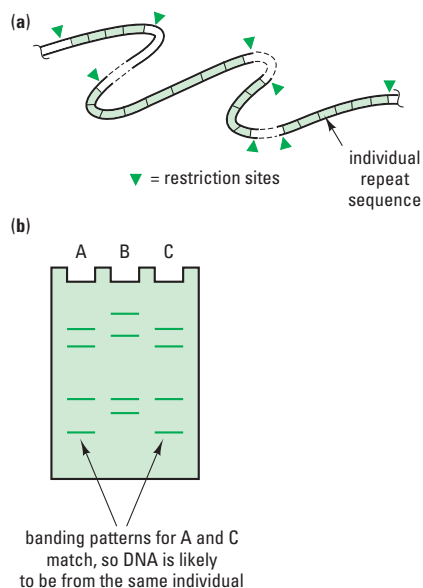


Fig. 68.3 Simplified representation of traditional DNA profiling: (a) tandem repeat sequences within DNA are cut using a restriction enzyme to yield fragments of different size; (b) these fragments are then separated by agarose gel electrophoresis on the basis of their size (M_r), giving a banding pattern that is a characteristic of the DNA used.

absence of a particular band on the gel, after PCR and electrophoresis). Such polymorphisms can be used to detect differences between dissimilar DNA sequences.

Applying PCR to DNA profiling

Genetic mapping and sequencing studies have led to the discovery of highly variable regions in the non-coding regions of DNA between different individuals. These hypervariable regions, sometimes termed ‘minisatellite DNA’, are found at many sites throughout the genome. Each minisatellite contains a defined sequence of nucleotides which is repeated a number of times in a tandem fashion (Fig. 68.3); the greater the number of repeats, the longer the minisatellite. The number of tandem repeats in any particular minisatellite varies from one person to another (i.e. they have a variable number of tandem repeats – VNTRs). This is exploited in identifying individuals on the basis of their DNA profile, by carrying out the following steps:

- 1. Extraction of DNA from cells of the individual** (for example, leukocytes, buccal cells, spermatozoa).
- 2. Digestion of the DNA with a restriction enzyme that cuts at sites other than those within the minisatellite**, to produce a series of restriction fragments of different M_r (Fig. 68.3(a)).
- 3. Electrophoresis, to separate the different restriction fragments** (Fig. 68.3(b)).
- 4. Southern blotting of the separated restriction fragments**, using probes specific to the particular VNTR/minisatellite.

The size of the fragments identified will depend on the number of tandem repeats that each fragment contains, and the pattern obtained in the Southern blot is characteristic of the individual being profiled (Fig. 68.3(b)).

PCR is widely used in DNA profiling in circumstances where there is a limited amount of starting material. By selecting suitable primers, highly variable regions can be amplified from very small amounts of DNA, and the information from several such regions is used to decide, with a very low chance of error, whether any two samples of DNA are from the same individual or not, for example in forensic science (Butler, 2009). Further developments include the use of short tandem repeat (STR) loci, summarized in Fig. 68.4.

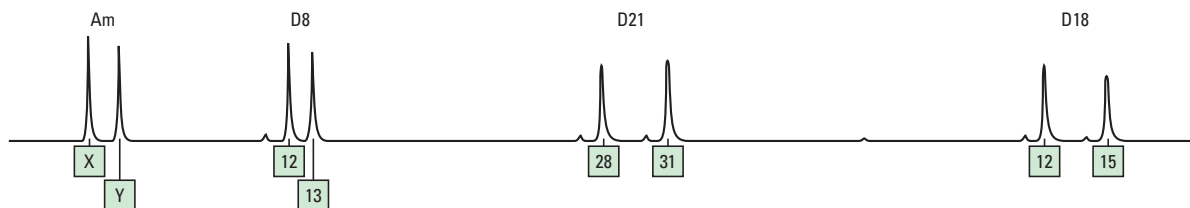


Fig. 68.4 Output of multilocus short tandem repeat (STR) analysis in contemporary DNA profiling. Highly polymorphic regions of the human chromosome with repeat sequences of 3–5 bases (STRs) are amplified by PCR and then separated/detected by capillary electrophoresis (p. 376). The profile above shows alleles (peaks) for four loci (shown in the top row of the figure), with two alleles for each locus (one inherited from each parent) – the numeral below each peak shows the number of repeats for the alleles D8, D21 and D18 while the Am (amelogenin) locus shows inheritance of alleles for X and Y chromosomes, which demonstrates that this profile is for a male. Typically, either 13 (US) or 11 (UK) STR loci are used to create an individual human DNA profile.

Understanding PCR acronyms – note that it is customary to abbreviate ‘reverse transcription PCR’ to ‘RT-PCR’, but to spell out ‘real-time PCR’ in full, to avoid any confusion. Similarly, ‘real-time, reverse transcription PCR’ can be abbreviated as ‘real-time RT-PCR’.

Using real-time PCR

Conventional PCR techniques rely on end-point detection of amplified product, for example, by electrophoretic separation and staining. However, such methods are time-consuming and are only semi-quantitative, since they are based predominantly on the detection of an amplified fragment (band) in a sample, rather than being designed to give exact information on its abundance (copy number). Quantitative analysis is only feasible during the early stages of PCR, where reagents are in excess and where the amount of amplified product is small, thereby avoiding the problems of product hybridisation, which would compete with primer binding. Real-time PCR enables the simultaneous amplification and quantification of template DNA in a sample by establishing the number of copies present by working in the early exponential phase of amplification. Currently, while several different formats are available for real-time PCR, all rely on the generation of a fluorescent signal, as described below.

Dye fluorescence

This is the simplest and least expensive approach. A fluorescent dye such as SYBR Green is included in the PCR mixture and the level of fluorescence is monitored as the reaction proceeds; since SYBR Green binds strongly to dsDNA, showing an enhancement in fluorescence of over 100-fold, any increase in fluorescence is directly proportional to the amount of dsDNA produced. Calibration is achieved by running a series of standards containing known amounts of dsDNA. This approach works well for optimized, single-PCR-product reactions where non-specific reactions are minimised.

Fluorescent reporter probes

Here a fluorescent ‘reporter’ dye is covalently bound to the 5’ end of an oligonucleotide probe while a ‘quencher’ group is attached to the 3’ end (Fig. 68.5). The probe is designed to hybridise to an internal region of the target sequence. During PCR, when the DNA polymerase molecule reaches the hybridised probe, the 5’ nuclease activity of the polymerase will cleave the reporter dye from the rest of the probe, causing an increase in fluorescence with each cycle that is in direct proportion to the amount of PCR product being formed, which is itself directly related to the original number of copies of the target DNA sequence. A commercial example is the TaqMan series of probes – while this approach is more accurate and reliable than dye fluorescence, it is also far more expensive, since a specific reporter probe must be synthesised for each target sequence. Other variants rely on changes in 3D conformation of the probe when it binds to the target sequence, causing an increase in fluorescence, for example Molecular Beacon probes.

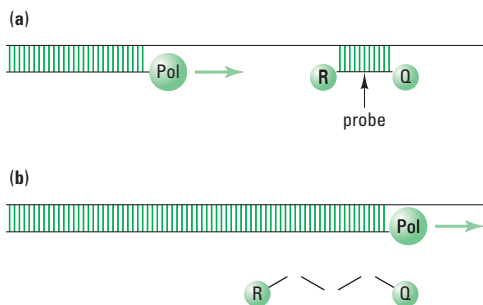


Fig. 68.5 Real-time PCR using a fluorescent reporter probe. In (a) the probe is bound to the target sequence while in (b) the 5’ nuclease activity of the DNA polymerase (Pol) has cleaved the reporter dye (R) and quencher (Q), resulting in fluorescence.

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STUDY EXERCISES

68.1 Investigate the role of magnesium ions in PCR.

Find out why optimising the Mg^{2+} concentration is important for successful amplification of target DNA in PCR.

68.2 Check your knowledge of primer design. List the principal factors that should be taken into account when designing primers for PCR.

68.3 Carry out a T_m calculation for a primer. For the following primer, what would be the temperature at which 50% of the molecules are annealed to their target sequence (i.e. T_m)?

5'–TGCATGGCTGGATTAGCG–3'

68.4 Consider how the risk of contamination can be reduced in PCR. List the most important practical steps that can be taken to reduce the possibility of contamination.

68.5 Find out what types of DNA analysis might be suitable for forensic examination of degraded DNA samples. In 1918 Tsar Nicholas of Russia, his wife

Tsarina Alexandra, and their five children were executed and buried in a shallow grave in Yekaterinburg, Russia. Samples from the bones of the family were analysed by the UK Forensic Science Service (FSS). What types of DNA-based tests would you expect to have been carried out to confirm that a family group was present in the grave?

68.6 Select appropriate primers for a specific PCR amplification. The sequence in the box below represents a region of DNA that is to be amplified by PCR.

Which of the following three pairs of primers is most suited to amplify a PCR product from the sequence in the box below?

- (a) 5'–TCGCTGAAGGACATGTTCG–3' and 5'–CGATCTAGGCGACATGTCC–3'
- (b) 5'–TCGCTGAAGGACATGTTCG–3' and 5'–TCCTAGGTCTCGACCGTA–3'
- (c) 5'–TCGCTGAAGGACATGTTCG–3' and 5'–GTCTCGACCGTAGCTAGCATC–3'

5'–TCGCTGAAGGACATGTTCGATGCTAGCTACGGTCGAGACGTAAGGACATGTCGGCTAGATCGC–3'

3'–AGCGACTTCCTGTACAGCTACGATCGATGCCAGCTCTGCATTCTGTACAGCGGATCTAGCG–5'

Answers to these study exercises are available at go.pearson.com/uk/he/resources

69 Genetic manipulation techniques

Complying with legislation for genetic manipulation – In the UK, the Genetically Modified Organisms (Contained Use) Regulations (2014) provide the regulatory framework for all research procedures involving the genetic modification of organisms.

Advances in the procedures used to manipulate nucleic acids *in vitro* have increased our understanding of the structure and function of genes at the molecular level (Chapter 67). Additionally, these techniques can be used to alter the genome of an organism (genetic manipulation, genetic engineering or gene cloning), for example, to create a bacterium capable of synthesising a foreign protein such as a potentially useful hormone or vaccine component, or a novel protein, through mutagenesis. The procedures are often termed ‘recombinant DNA technology’, since they involve the creation of novel combinations of DNA (i.e. recombinant DNA) under controlled laboratory conditions.

KEY POINT While genetic manipulation must be carried out under strict containment, in accordance with appropriate legislation, the procedures involved in the isolation, amplification, recombination and cloning of DNA are often used at undergraduate level, to illustrate the general features of the techniques.

Definitions

Bacteriophage (phage) – a bacterial virus. Useful as they possess a means of penetrating the bacterial cell, enabling entry of recombinant DNA.

Cloning vector – a DNA molecule that can be combined with foreign DNA *in vitro*, then introduced and maintained in a host cell.

Cosmids – hybrid plasmid cloning vectors containing the *cos* (cohesive) sites from phage λ , enabling *in vitro* packaging into phage capsids. Useful for cloning large segments of DNA, typically up to 50 kbp.

Plasmids – circular molecules of DNA capable of autonomous replication within a bacterial cell. Can be isolated, manipulated and reintroduced into bacterial cells.

Subcloning – procedures that isolate and characterise smaller portions of a particular DNA sequence, e.g. during the search for a specific gene.

Transfection – in bacteria: uptake of viral nucleic acid; in eukaryotes: uptake of any naked, foreign DNA.

Transformation – in bacteria: stable incorporation of external DNA, e.g. a plasmid; in eukaryotes: the conversion of a cell culture of finite life to a continuous (immortal) cell line (this also occurs in cancers).

Basic principles

Gene cloning involves several steps:

1. **Isolation of the DNA sequence (gene) of interest** from the genome of an organism, or from a gene library. This usually involves either (i) DNA purification (Chapter 60), followed by enzymic digestion or mechanical fragmentation, to liberate the target DNA sequence, or (ii) PCR amplification (Chapter 68) of the target DNA sequence. Box 67.1 explains how to isolate plasmid DNA from bacteria.
2. **Creation of an artificial recombinant DNA molecule (rDNA)**, by inserting the target sequence into a DNA molecule capable of replicating in a host cell, i.e. a ‘cloning vector’. Suitable cloning vectors for bacterial cells include plasmids, bacteriophages and cosmids, as described below.
3. **Introduction of the recombinant DNA molecule into a suitable host**, for example *E. coli*. The process is termed transformation when a plasmid is used, or transfection for viral nucleic acid.
4. **Selection and growth of the transformed (or transfected) cell**, using the techniques of cell culture. Since a single transformed host cell can be grown to give a clone of genetically identical cells, each carrying the target DNA of interest, the technique is often referred to as ‘gene cloning’, or molecular cloning.
5. **Expression of the target DNA by the transformed cell, generating a recombinant protein product**. Such recombinant proteins are used in medical science (for example, recombinant human insulin in the treatment of diabetes) and biotechnology (for example, proteases in the food industry).
6. **Where appropriate, modification of the target DNA sequence by site-directed mutagenesis**. These methods can be used to make specific changes to the target sequence, enabling the investigation of such changes on transcription, translation and protein function as a key aspect of ‘protein engineering’.

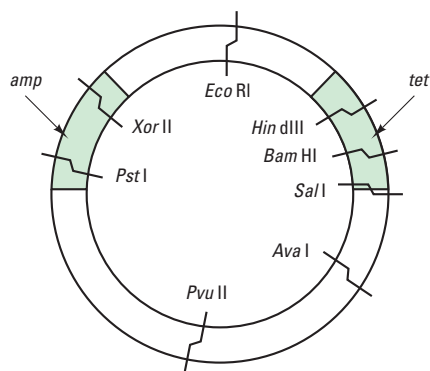


Fig. 69.1 Restriction map of the plasmid pBR322. The position of individual restriction sites is shown together with the genes for ampicillin resistance (*amp*) and tetracycline resistance (*tet*).

Definition

Type II restriction endonucleases – intracellular enzymes, produced by certain strains of bacteria: their function is to restrict the growth of phages within the cell by cutting phage DNA at particular sites (host DNA is protected by methylation at these sites).

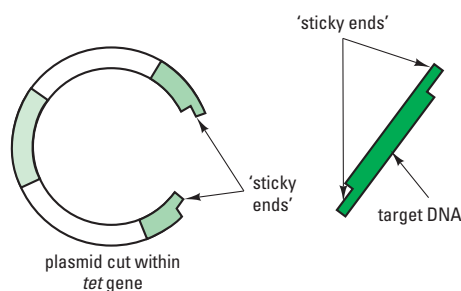


Fig. 69.2 Restriction of plasmid pBR322 and foreign DNA with *Hin* dIII.

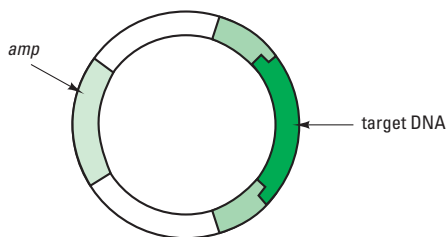


Fig. 69.3 Annealing and ligation of plasmid pBR322 and target DNA to give a recombinant plasmid which confers resistance to ampicillin only. As target DNA has been inserted within the *tet* gene (Fig. 69.1), the gene is now discontinuous and inactive.

Using DNA-manipulating enzymes

Restriction enzymes

Type II restriction endonucleases can be used to produce linear fragments of DNA with either single-stranded 'sticky' ends or 'blunt' ends (Fig. 67.1): most commercial preparations of restriction enzymes will completely cleave a sample of DNA, producing a 'restriction digest', within 1 h at 37 °C. The position of individual restriction sites can be used to create a diagnostic restriction map for a particular molecule, for example a plasmid cloning vector (Fig. 69.1). An important additional feature is that, under appropriate conditions, the sticky ends of any two restriction fragments cut with the same enzyme (Fig. 69.2) can anneal (base pair), because of the formation of hydrogen bonds between individual bases within this region, allowing them to be joined together (ligated), irrespective of the sources of the two restriction fragments. Use online tools to identify useful restriction enzymes for a particular target sequence (for example, see: <http://nc2.neb.com/NEBcutter2/>).

DNA ligase

To construct a recombinant DNA molecule, a suitable cloning vector must be ligated to the DNA fragment to be cloned: this is performed using another microbial enzyme, DNA ligase (usually obtained from T4-infected *E. coli*). This ATP-dependent enzyme is capable of forming covalent phosphodiester bonds between annealed DNA molecules, thereby creating recombinant DNA (Fig. 69.3). When the two molecules involved are the cloning vector and the target DNA, the size of the recombinant molecule can be predicted (for example, a plasmid of 4.5 kbp, plus a target DNA fragment of 2.5 kbp will give a recombinant molecule of 7 kbp), allowing separation and recovery by agarose gel electrophoresis (pp. 514, 515). Ligation is usually carried out at lower temperatures (to encourage annealing), over an extended time period of several hours (to allow the enzyme to operate), for example, overnight incubation at $\approx 16^\circ\text{C}$. The volume of the ligation mixture is kept as low as possible (typically, $<10\ \mu\text{L}$), with approximately a 2:1 ratio of target DNA to vector DNA, to encourage annealing between the two different types of DNA molecule and to reduce the chance of circularisation of vector or target DNA. Treatment of target DNA with alkaline phosphatase reduces the risk of self-ligation.

Choosing a suitable cloning vector

The following features should be considered:

- **ease of purification** – for example, reliable procedures have been developed to allow plasmid DNA to be extracted and purified from bacterial cells
- **efficiency of insertion** of the recombinant cloning vector into a new host cell
- **presence of single copies of suitable restriction sites**
- **presence of selectable markers** (for example, 'reporter genes', p. 532)
- **size of the DNA insert to be cloned**
- **copy number of the cloning vector** in the host cell.

Plasmids

The simplest bacterial cloning vectors are those based on small plasmids (Chapter 66). One of the first cloning vectors to be developed for *E. coli* was pBR322, a genetically engineered plasmid of 4.4 kbp containing an origin

Understanding Gibson assembly cloning

– this development offers an alternative to the conventional approach, where fragments cut with the same restriction enzyme are ligated. Here, fragments with overlapping terminal sequences are converted to ‘sticky end’ format using a single-strand exonuclease prior to ligation (for further details, see Codex, 2021).

Understanding the nomenclature for plasmid cloning vectors

– pBR322 is named according to standard rules: ‘p’ indicates the vector is a plasmid, ‘BR’ identifies the researchers Bolivar and Rodriguez and ‘322’ is the specific code given to this plasmid, to distinguish it from others developed by the same workers.

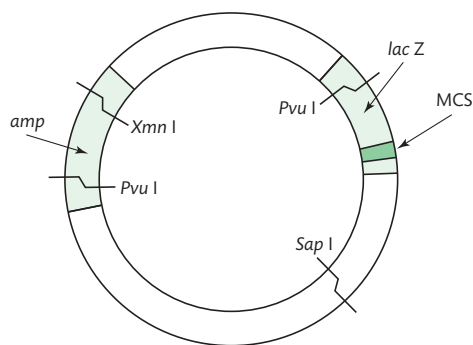


Fig. 69.4 Restriction map of the plasmid pUC19. The position of some individual restriction sites is shown together with the genes for ampicillin resistance (*amp*) and β -galactosidase (*lacZ*). MCS = multiple cloning site for 40 restriction enzymes within the *lacZ* gene.

of replication, two antibiotic resistance genes and single sites for a range of restriction enzymes (Fig. 69.1). Subsequently, other plasmid cloning vectors have been developed, with additional features: for example, plasmids of the pUC series (Fig. 69.4) are now widely used for transformation of *E. coli*, having the following advantages over earlier types:

- **high copy number:** several hundred identical copies of the plasmid may be present in each cell, giving improved yield of plasmid DNA
- **single-step selection of recombinants** using the *lacZ'* gene (p. 533)
- **clustering of restriction sites** within a short region of the *lacZ'* gene – restriction using two enzymes (a double digest) cuts a small fragment from this multiple cloning site or ‘polylinker’, producing a cleaved plasmid with two different ‘sticky ends’ (for example, *Eco* RI at one end and *Hin* dIII at the other), allowing complementary fragments of target DNA to be ligated in a particular orientation (directional cloning).

The pET series of plasmid cloning vectors from Novagen (Fig. 69.5) is widely used, since they offer several additional advantages, including:

- **high levels of protein expression**, as the cloned DNA is under the control of the T7 promoter and the lac operon system
- **specialised cloning vectors and hosts** for the production of soluble proteins, disulfide bond formation, protein export, etc.
- **the attachment of a short histidine ‘tag’** on the end of an expressed protein, to enable the protein to be purified by immobilised metal affinity chromatography (IMAC), as described on p. 355.

Phages

Bacteriophage cloning vectors can offer several advantages over plasmids:

- some phages have been engineered to carry larger fragments of foreign DNA – plasmid cloning vectors work best with short inserts of 1–2 kbp, while some phage vectors can accept 10 kbp of foreign DNA
- a recombinant phage has a ready-made mechanism for transferring rDNA to a new host cell: the process is broadly similar to phage transduction, described on pp. 503–4.

A number of cloning vectors have been developed from *E. coli* phage λ (genome = 49 kbp of dsDNA in linear form, Fig. 69.6). One example is λ ZAPII, an insertion vector capable of accepting up to 10 kbp of additional (foreign) DNA and with the *lacZ'* gene and multiple cloning site for selection. Another is λ EMBL4, a replacement cloning vector, where part of the vector DNA is removed and replaced by foreign DNA (up to 23 kbp) during cloning.

Most cloning vectors based on phage λ are virulent, released by lysis of the infected cell at the end of the replication phase: such vectors are cloned using standard techniques for phages, for example, as plaques of lysis on a ‘lawn’ of bacteria growing on an agar-based medium (p. 237). Note that suitable strains of *E. coli* must be used for each of the various cloning vectors – for example, vectors containing *lacZ'* can only be used with those *E. coli* strains that are deficient in this part of their genome (*lacZ'* codes for the α -component of the enzyme β -galactosidase, present in wild-type *E. coli*). In some instances, a second phage (a ‘helper phage’) must be used along with the cloning vector, to carry out some of the functions removed

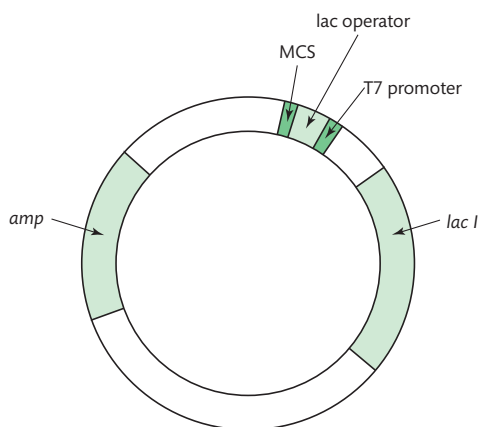


Fig. 69.5 Map of a pET plasmid cloning vector (Novagen) showing the location of the genes for ampicillin resistance (*amp*) and lactose repressor protein (*lacI*) which controls expression of the T7 promoter via the lac operator (binding of the Lac repressor protein is countered by addition of isothiopropylgalactoside, IPTG). Expression of a gene cloned into the multiple cloning site (MCS) can thus be controlled, since DNA transcription only occurs when IPTG is added to the growth medium (inducible expression system).

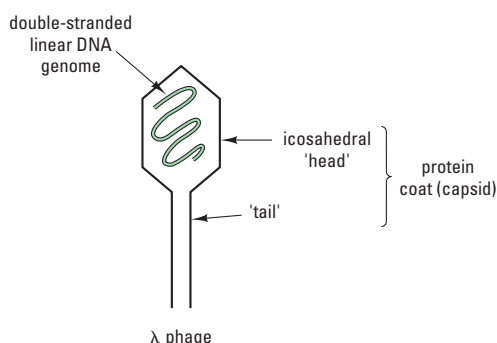


Fig. 69.6 Bacteriophage λ .

Definition

Phagemid – a recombinant entity created by fusion of a plasmid with the origin of replication of a phage: can be propagated and packaged into phage particles with the aid of a 'helper' phage.

from the vector genome, such as phage packaging and assembly. In some instances, more specialised phage cloning vectors may be required, for example, single-stranded M13mp19, used for sequencing.

Cosmids and other cloning vectors

Cosmid vectors contain the single-stranded cohesive terminal sequences from λ phage (so-called 'cos' sites) inserted into a plasmid cloning vector. They can be used to clone up to 40 kbp of foreign DNA, using the 'cos' sites to allow *in vitro* packaging into the λ phage coat: such large fragments are required for the production of gene libraries, as described below. Other hybrid cloning vectors have been produced by the fusion of a phage and a plasmid – these 'phagemids' can be manipulated as plasmids for ease of DNA uptake, or converted to phage-like form for ease of storage.

Creating a gene library

For viruses with small genomes, an individual gene may be identified by hybridisation of a single fragment with a suitable probe, following digestion with a particular restriction enzyme (Chapter 67). However, for most genomes, such a digest would contain a very complex mixture of fragments and a 'clone library' must be created. The isolation and identification of a particular gene are then carried out by screening individual clones from this library. Two types of clone library can be used:

1. **A genomic library** – prepared from the entire genome of the organism under study. The genome is fragmented to give overlapping fragments, for example by partial restriction (incomplete digestion at low temperature), or by mechanical shearing. Individual fragments are then incorporated into a suitable cloning vector, such as a cosmid, to create a vector library (a cosmid library). Each recombinant vector is then transferred to a separate host cell, which is cultured, giving a collection of transformants that represents the clone library. This approach is sometimes termed 'shotgun cloning'.
2. **A cDNA library** (cDNA = complementary, or copy DNA) – prepared by converting mRNA into DNA using the retroviral enzyme reverse transcriptase. Such a library consists only of genes expressed in those cells used to create the library, making individual genes easier to locate and identify, since the number of clones represented is usually smaller than for a genomic library. Another feature of cDNA is that it is complementary to the processed transcript (mRNA), so it contains no information on non-coding regions (introns) or transcriptional sequences (promoter regions, etc.).

Transferring rDNA to a suitable host cell

Once a recombinant vector has been produced *in vitro*, it must be introduced into a suitable host cell. Packaged phage cloning vectors have a built-in transfer mechanism, while naked phage DNA and plasmids must be introduced by treatments that cause a temporary increase in membrane permeability resulting in transformation of the host cell. Treatments include:

- **physicochemical shock treatment** – Box 69.1 gives details of a typical procedure using CaCl_2 and heat shock treatment to transform *E. coli* with the plasmid pUC19

Screening clones – a particular gene can be detected using Southern blotting (pp. 516, 526), or the expressed gene product (protein) may be detected by a suitable method, e.g. an immunoassay. While it is relatively simple to describe the screening process, finding a specific gene (= clone) in a clone library is likely to be a time-consuming process.

Using cloned cDNA – information from the sequence of a particular cDNA clone may be useful in designing a suitable oligonucleotide probe to locate a particular gene in a genomic library.

Table 69.1 Examples of genes used to detect transformants.

Gene	Product/assay
<i>lacZ/lacZ'</i>	β -galactosidase/chromogenic substrate (e.g. X-GAL) or fluorogenic substrate (e.g. MU-GAL)
<i>uidA</i>	β -glucuronidase/chromogenic substrate (e.g. X-GLUC/X-GUR) or fluorogenic substrate (e.g. MU-GLUC/MU-GUR)
<i>Lux</i>	luciferase/bioluminescence in the presence of luciferin
<i>bla/amp</i>	β -lactamase/resistance to ampicillin
<i>Cat</i>	chloramphenicol acetyltransferase/resistance to chloramphenicol
<i>gfp</i>	green fluorescent protein from <i>Aequorea victoria</i> , visualised under UV light

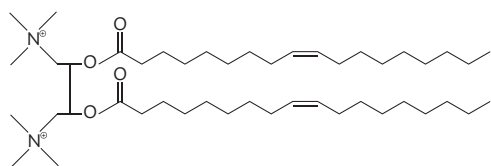


Fig. 69.7 General structure of a synthetic cationic lipid, for example, Tfx.

- **electroporation** – cells or protoplasts are subjected to electric shock treatment (typically, $>10\text{kV cm}^{-1}$) for very short periods ($<10\text{ms}$)
- **micro-injection treatments** – used with animal and plant cells, either via a microsyringe, DNA-coated microprojectiles ('biolistics') or cationic lipids (Fig. 69.7) which form lipid–DNA complexes that fuse with cell membranes and allow DNA entry.
- ***Agrobacterium*-mediated transfer** – this plant pathogenic bacterium contains a plasmid (Ti) that can be used as a cloning vector in a range of plants.

Selecting and detecting transformants

As the efficiency of transformation is often very low, many of the plasmid cloning vectors used in genetic engineering carry genes coding for antibiotic resistance, for example pBR322 carries separate genes for ampicillin resistance, *amp*, and tetracycline resistance, *tet* (Fig. 69.1). These genes act as 'markers' for the cloning vector. One gene (*amp*) can be used to select for transformants, which would form colonies on an agar-based medium containing the antibiotic, while non-transformed (ampicillin-sensitive) cells would be killed. The other gene (*tet*) can be used as a marker for the recombinant plasmid vector, since ligation of the target sequence into this gene causes insertional inactivation (Fig. 69.3). Thus, cells transformed with the recombinant plasmid will be resistant to ampicillin only, while cells transformed with the recircularised ('native') plasmid will be resistant to both antibiotics. These two types of transformant can be distinguished using replica plating (see p. 502). For those genes where the product is an enzyme, the presence or absence of the functional gene can be assessed using a suitable substrate. For example, the insertional inactivation of *lacZ'* can be detected by including a suitable inducer of β -galactosidase (for example, isopropylthiogalactoside, IPTG) and the chromogenic substrate 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-GAL) within the agar medium: a transformant colony derived from the native plasmid will be blue-green while a transformant containing the recombinant molecule (inactive *lacZ'*) will grow to produce a white colony (so called 'blue-white screening'). A number of other examples are given in Table 69.1.

Using site-directed mutagenesis and genome editing techniques

These methods are used to investigate the structure and function of proteins produced following the introduction of specific changes to the sequence of bases in a particular gene that has been cloned and sequenced. Site-directed mutational changes that can be introduced include base insertions, deletions and substitutions, offering an alternative to conventional chemical mutagenesis (Box 34.3). One *in vitro* technique is to use synthetic oligonucleotide primers (p. 522) containing the required mutational change within a sequence that is otherwise complementary to the target sequence, with primer extension carried out by DNA polymerase, typically using PCR to amplify the mutant gene sequence. During the PCR process, the desired mutation is introduced into the amplicon at one of the two primer sites, replacing the original sequence during the amplification process. The mutation-carrying amplicon can then be isolated either using a restriction enzyme that will digest the 'parental' amplicon, or by gel

Box 69.1 How to transform *E. coli* and select transformants

The following procedure illustrates the principal stages of the process: the efficiency of transformation will depend on your choice of *E. coli* strain and plasmid, and on the handling procedures you use prior to and during the process. Sterile equipment and appropriate technique (Chapter 33) are required at all times.

- 1. Grow the cells under appropriate conditions:** the best results are often obtained by using actively growing, mid-log phase cells, rather than cells that have been grown to stationary phase (p. 243). Actively growing cells can be stored under appropriate conditions, e.g. as a 'frozen stock' – in suspension in concentrated glycerol at -85°C .
- 2. Induce cell competence by transfer to a suitable sterile transformation buffer:** this would normally contain divalent and monovalent salts, e.g. a salt solution containing CaCl_2 at $50\text{--}100\text{ mmol L}^{-1}$, plus KCl and MnCl_2 at $10\text{--}20\text{ mmol L}^{-1}$. Keep cells on ice in this solution for 10–15 min, to encourage the binding of plasmid DNA, added at a later stage.
- 3. Add reagents to increase the permeability of cellular membranes:** typically add dimethyl sulfoxide (DMSO, at up to 7%v/v) and dithiothreitol (DTT at up to 0.2 mmol L^{-1}), and incubate on ice for a few minutes. DMSO is readily oxidised, reducing the effectiveness of the transformation procedure, and should be stored at -80°C when not in use, to minimise oxidation.
- 4. Add plasmid DNA:** typically at $10\text{--}1000\text{ ng}$ per transformation. Maintain on ice for at least 10 min in a minimal volume of solution, to allow the plasmid to become associated with the cell surface.
- 5. Heat shock the cells:** briefly raise the temperature to $42\text{--}45^{\circ}\text{C}$ for 60–120 s, then return to ice for a few minutes. It is important that this treatment gives a *rapid* change in temperature – use a small, thin-walled (disposable) sterile plastic tube containing the minimum volume of solution to maximise the rate of temperature change.
- 6. Allow the cells to recover from heat shock and to express any new genes** (for example, to synthesise enzymes conferring antibiotic resistance in transformed cells): add sterile nutrient broth (e.g. 1 mL) and then incubate at 37°C for up to 60 min.
- 7. Plate onto an appropriate medium, to allow selection and detection of transformants:** for example, using pUC19, surface spread the suspension onto a medium containing ampicillin (at 25 mg L^{-1}), plus IPTG (at 15 mg L^{-1}) and X-GAL (at 25 mg L^{-1}), to detect the *lacZ'* gene product, as described in the text above.

Recognising transformants – after plating bacteria onto medium containing ampicillin, you may notice a few small 'satellite' colonies surrounding a single larger (transformant) colony. These satellite colonies are derived from non-transformed cells which survive to the breakdown of antibiotic in the medium around the transformant colony, and should not be selected for subculture.

electrophoresis. It is then inserted into a suitable host cell using the standard genetic engineering techniques described above.

Recently, *in vivo* techniques have been developed that enable gene editing without the need for isolation and manipulation of DNA in the laboratory. The most important of these makes use of the bacterial CRISPR-Cas9 system where **Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)** sequences are cleaved by **CRISPR-associated (Cas)** enzymes, serving to prevent the replication of target phages in bacterial cells. Gene editing makes use of the Cas9 nuclease, delivered together with a synthetic 'guide RNA' that has a sequence complementary to that of the target region of the genome of the cell, thereby enabling Cas9 to cut the DNA at this specific point. Cas9-mediated cuts can lead to gene inactivation, deletion or insertion, depending upon the specifics of the process. Because *in vivo* gene editing techniques result in permanent changes to the genome of the recipient cell, they give rise to bioethical concerns, especially in relation to their possible use with human germline cells and embryos (see also Chapter 30). For further practical details, see Luo (2019).

Text references and Sources for further study

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STUDY EXERCISES

69.1 Explain the terminology of restriction enzymes.

Why were *Hin* dIII and *Eco* RI given these names?

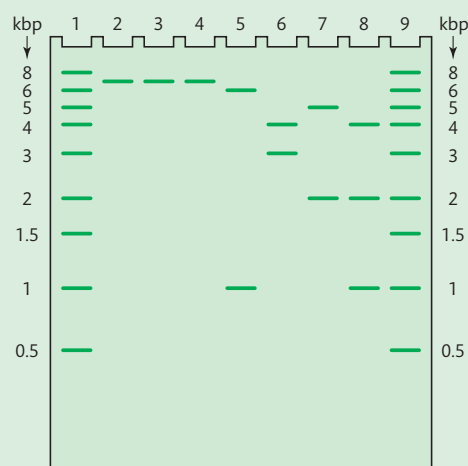
69.2 Explain the purpose of the various stages involved in the extraction and purification of DNA.

Having read through this chapter, briefly describe the main purpose of each of the following reagents within a DNA extraction protocol, and rearrange them into their order of use within the procedure:

- anion exchange resin column chromatography;
- incubation in 70% v/v ethanol: water;
- ribonuclease and proteinase digestion;
- incubation with lysozyme/EDTA;
- sodium dodecyl sulfate incubation;
- NaOH treatment, followed by neutralisation with potassium acetate.

69.3 Construct a restriction map.

The figure alongside represents an electrophoretic separation of plasmid pRD, digested with different restriction enzymes, both individually and in combination. All of the enzymes cut the plasmid only once. Use this information to construct a simple restriction map of the plasmid. Key to lanes: 1 = DNA standards (0.5–8 kbp); 2 = *Eco* RI; 3 = *Hin* dIII; 4 = *Pvu* II; 5 = *Eco* RI and *Hin* dIII; 6 = *Eco* RI and *Pvu* II; 7 = *Hin* dIII and *Pvu* II; 8 = *Eco* RI, *Hin* dIII and *Pvu* II; 9 = DNA standards (0.5–8 kbp).

**69.4 Calculate transformation efficiency.**

A volume of 100 μL of *E. coli* cells was transformed using 20 μL of plasmid DNA (prepared at $0.1 \text{ ng } \mu\text{L}^{-1}$) and then made up to a final volume of 500 μL in buffer. A 10-fold dilution of this suspension was prepared and 200 μL of this dilution was surface spread onto a suitable medium containing an antibiotic to select for transformants, giving 180 colonies on the spread plate after overnight incubation. What is the transformation efficiency, expressed as the number of transformants (colony-forming units, CFU) per μg DNA? (Give your answer in exponential notation, to three significant figures.)

Answers to these study exercises are available at go.pearson.com/uk/he/resources

70 Applying bioinformatics

Definitions

Genome – the entire complement of genetic information (coding and non-coding DNA) of an organism.

Metabolome – all of the metabolites (low molecular mass biomolecules) of an organism.

Proteome – the expressed protein complement of the genome.

Secretome – all of the secreted proteins from an organism.

Transcriptome – the complement of -mRNAs transcribed from the genome, weighted by the expression level of each RNA.

Note – that terms ending in 'ome' can be used to describe disciplines by changing the ending to 'omics'.

Definition

Chemoinformatics – the application of information technology to chemical data and information, especially in the field of drug design/discovery.

Understanding the scale of primary sequence databases – the EBI-NCBI-DDBJ databases holds many millions of nucleic acid sequence records from many thousands of different organisms.

Complex polymers such as nucleic acids and proteins contain a large amount of information within their structures, particularly with respect to the sequence of constituent sub-units – nucleotide base and amino acids, respectively. Visual analysis and interpretation of this sequence information is impractical for all but the smallest polynucleotide or polypeptide fragments. Bioinformatics is a term used to describe the application of computer-based techniques in the biological sciences and, in particular, to the analysis of base sequences in nucleic acids (genomics, transcriptomics) and to the amino acid sequences of proteins predicted from nucleic acid sequence data (proteomics), as well as investigation of nucleic acid and protein structure.

KEY POINT Computer-based analysis of information in complex macromolecules typically compares sequence data for all or part of a specific biomolecule with that of others in a database, to determine similarities and differences and to predict spatial relationships within macromolecules.

Table 70.1 gives details of some of the most important databases accessible via the Web.

A range of programs and packages can be used to analyse nucleic acid and protein sequences. Many programs can be used online, while others can be downloaded and used offline. In most instances, the information and software are freely available. The large number of different programs available is a reflection of the various algorithms used for analysis; such programs are updated in parallel with advances in computer hardware/software. When using a new bioinformatics program, you should make use of the help facilities to familiarise yourself with the operating principles.

Using primary sequence databases

The major databases holding primary sequence information for nucleic acids and proteins are operated by the European Bioinformatics Institute (EBI) of the European Molecular Biology Laboratory, EMBL (at <https://www.ebi.ac.uk>), the National Center for Biotechnology Information (NCBI) GenBank database (at <https://www.ncbi.nlm.nih.gov/genbank/>) and the DNA Data Bank of Japan, DDBJ (at <http://www.ddbj.nig.ac.jp>). These databases hold comprehensive information submitted by researchers and sequencing groups across the world, with collaborative data sharing on a daily basis, as part of the International Nucleotide Sequence Database Collaboration (<http://www.insdc.org>).

Nucleic acid analysis

A typical application would be to investigate whether a nucleic acid sequence obtained, for example, as part of a cloning project (p. 529), has been reported previously. Table 70.1 gives details of URLs for the major nucleic acid databases. These databases allow you to:

- **enter a particular nucleic acid sequence and edit the sequence**, if required
- **find and retrieve a nucleotide sequence from the database** using a keyword, author name, organism name or an accession number, where known

Table 70.1 Some useful databases and other Internet locations relevant to bioinformatics.

Major nucleic acid sequence databases	
EMBL-EBI European Nucleotide Archive	https://www.ebi.ac.uk/ena
NCBI GenBank	https://www.ncbi.nlm.nih.gov/genbank
Major protein sequence databases	
UniProt (universal protein resource)	https://www.uniprot.org
PIR (protein information resource)	https://proteininformationresource.org
Other bioinformatics databases	
Online Bioinformatics Resource Center (links to databases and resources)	https://www.hsls.pitt.edu/obrc/ https://www.sanger.ac.uk/
Wellcome Sanger Institute (links to databases)	
PROSITE (protein motifs)	https://prosite.expasy.org/
PRINTS (protein fingerprints)	http://130.88.97.239/PRINTS/index.php
ExPASy proteomics resource	https://www.expasy.org
PDB (Protein Data Bank)	https://www.rcsb.org/pdb
Array Express (Microarrays)	https://www.ebi.ac.uk/arrayexpress
MEROPS (protease/peptidase database)	https://www.ebi.ac.uk/merops/
Transport protein database	http://tcdb.org/
CBS prediction servers (various databases)	http://www.cbs.dtu.dk/services/
PubMed (literature database)	https://pubmed.ncbi.nlm.nih.gov

Example Y10510 is the accession number for a human mRNA sequence for the CD67S protein in the EMBL-EBI database.

Searching primary databases – a search of the EMBL-EBI nucleic acid database can be carried out using BLAST (Fig. 70.1) to locate related sequences.

Definition

Palindrome – a sequence reading the same in both directions, e.g. GACCAG. These are often sites at which restriction enzymes cleave DNA (see pp. 512–13).

- **translate a nucleotide sequence into an amino acid sequence and vice versa**
- **search for any resemblance between a particular sequence and sequences within the database**, for example by comparing and aligning the sequence for several nucleic acids or proteins, to identify regions of sequence similarity (see Fig. 70.1)
- **carry out phylogenetic analysis**, constructing ‘ancestry trees’ to show the most likely evolutionary relationships between sequences from various organisms.

Other programs allow you to analyse nucleic acid sequence information in more detail, for example:

- **to assemble/align sequence fragments into contiguous sequences**, also known as ‘contigs’. This type of alignment is particularly useful for sets of nucleic acid fragments from a clone library (p. 532)
- **to search for structural and functional ‘motifs’ (repeating patterns) within the nucleic acid sequence**, including palindromes, restriction sites (pp. 512–13), likely promoter or control sites and regions that might form secondary structures, such as hairpin loops
- **to identify likely coding regions (for example open reading frames, exons) and non-coding regions (e.g. introns, repeat sequences) in eukaryotic DNA, ‘start’ and ‘stop’ codons, etc.** The starting point for this analysis is the creation of a ‘six frame translation’ of the target sequence (see Fig. 70.2)

```

H. sapiens      atgtatggcatcgagaatgaagtcttcctgagccttccatgtatcctcaatgccgggg
               ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
R. norvegicus  atgtacggcatcgagaacgaagtcttcctcagtcctccgtgatccttaatgctcgggg

```

Fig. 70.1 Representative output from a BLAST (basic local alignment search tool) alignment search for DNA sequences from part of the lactate dehydrogenase genes of *Homo sapiens* and *Rattus norvegicus*. Here, identical bases are shown in black, and non-identical bases are shown in green. This region shows 49 identical bases out of 59, i.e. $49 \div 59 \times 100 = 83\%$ similarity (to the nearest integer).

Remembering which is which – INTrons (non-coding sequences) INTerrupt coding sequences while EXons are EXpressed (translated into an amino acid sequence).

Using bioinformatics databases – many of the database websites are rather complex and newcomers may find them a little difficult to navigate, especially if you are just browsing. The best approach is to try out the online tutorials, to get a feel for how the website operates. If the URL changes, use a search engine to locate the new website.

Understanding the basic terminology of biopolymer structure:

- **primary structure** – sequence of amino acids along a polypeptide;
- **secondary structure** – regular repeating structural motifs (α -helix and β -sheet), stabilised by hydrogen bonds;
- **tertiary structure** – overall 3D form of the polypeptide;
- **quaternary structure** – where more than one polypeptide subunits form a functional complex.

- **to simulate *in vitro* manipulation and cloning procedures**, including the preparation of restriction digests, designing oligonucleotide probes and primers, and simulating PCR (p. 522).

Analysis of proteins and polypeptides

The principal protein sequence databases are UniProt in Europe, together with PIR in the USA: Table 70.1 gives their current Web locations. These databases can be searched using a range of programs equivalent to those described above for nucleic acid analysis, allowing the operator to:

- **locate and retrieve a particular protein or amino acid sequence** from the database by keyword, author name, organism name or accession number
- **enter and edit (cut and paste) a particular amino acid sequence**, or convert a nucleotide sequence into an amino acid sequence, and vice versa
- **search the database for sequences** with a perfect match, or for similarities in primary or secondary structure at a specified level of homology; such searches may be more informative than those based on DNA homology, since protein sequence information (based on over 20 different amino acid residues, p. 423) is more complex than nucleic acid sequence information (based on four different nucleotides, p. 446)
- **compare and align the amino acid sequences of several proteins**, to identify particular regions of similarity and dissimilarity.

Using other bioinformatics databases

Pattern recognition databases for proteins

These have been created using primary sequence data, to provide information on the patterns identified within proteins. The representative examples given in Table 70.1 include the PROSITE and PRINTS databases, which identify protein families by their diagnostic ‘signature’ motifs, and

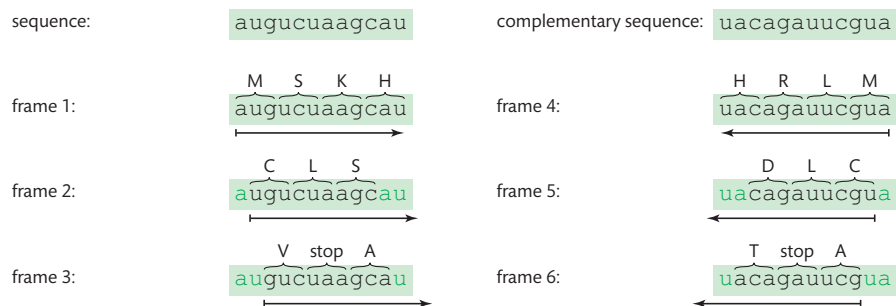


Fig. 70.2 ‘Six frame translation’ of a DNA sequence. The triplet codons corresponding to each amino acid are given in Table 67.1 (p. 511) and the single letter amino acid code is explained in Table 57.1. Note that the complementary sequence is translated in the reverse direction to the original sequence.

the Expert Protein Analysis System (ExPASy) website, which provides a gateway to the general analysis of protein structure, including 3D imaging, while the CBS Prediction Servers can be used to identify particular motifs, as well as other aspects of genome analysis.

Many of these ‘second-level’ databases provide tools and software that can be used for a range of purposes, including:

- **assembling overlapping peptide fragments**, based on their amino acid sequences, for example during sequence analysis of an individual protein.
- **predicting secondary structure from a primary sequence**, identifying regions most likely to exist as *α*-helices or *β*-sheets, etc.
- **searching for motifs and patterns within a sequence** that might provide information on the origins and/or possible function(s) of the protein
- **predicting potential sites of post-translational modification**
- **modelling the likely 3D structure of the protein**, identifying likely membrane-spanning regions, active sites and other tertiary and quaternary features
- **investigating properties such as hydropathy**, solvent accessibility and antigenicity
- **predicting and modelling the effects of changes in primary or secondary structure** on protein characteristics and 3D organisation.

Definition

Hydropathy – hydrophobic interactions within a biomolecule; often important in determining folding and 3D structure.

Using bioinformatics in drug design

– protein databases provide chemoinformatic information that can be used to locate new therapeutic targets, e.g. when designing novel antimicrobial agents.

Example PyMOL is a molecular graphics program that enables the user to visualise the structure of biomolecules – it can be obtained from the following website: <http://pymol.sourceforge.net/>

Applying next-generation sequencing – high throughput techniques (p. 520) have revolutionised the study of genomics, by providing rapid and less expensive approaches than traditional sequencing.

Protein-specific databases

Several databases have been created to collect together information on a particular class of proteins – many are devoted to particular enzymes or groups of enzymes. An example of a second-level protein database is that devoted to the analysis of enzymes that degrade, modify or create glycosidic bonds, the carbohydrate-active enzymes (CAZy database, at <http://www.cazy.org>). This website brings together information on the various families of enzymes that interact with carbohydrates, enabling researchers to compare sequences against known catalytic and carbohydrate-binding modules, arranged by enzyme class and by organism. Links to related websites are also included. Another example is the MEROPS protease database, which provides a structure-based catalogue of proteases and peptidases, giving amino acid and nucleotide sequences, classification and nomenclature, plus links to relevant literature through PubMed. If you are looking for information on a particular enzyme or other protein, it may be worth using a Web search engine to see whether a specific database has been created.

Protein structure

The Protein Data Bank (PDB) is the worldwide repository for 3D structures of proteins (<https://www.pdb.org/>), also serving as a general portal for structural information on biological macromolecules. The database can be searched by identity number (for example 1 ALB is the code for an adipocyte lipid-binding protein) or by name (note that this is a US resource, so using the US spelling ‘hemoglobin’ locates more structures than using the UK spelling). Structures can be viewed in a variety of formats, including those compatible with molecular graphics software such as PyMOL, RasMol and Chime. Additional information includes secondary structure (helices, sheets, hairpins, etc.), crystallographic data, geometry, etc., plus links to related sites. Figure 70.3 shows an example of a typical output – in this instance,



Fig. 70.3 Representation of the 3D structure of a pectate lyase from *Cellvibrio japonicus*. This is a solid ribbon display, with helices and sheets shown in green. The cleft, in which the plant polysaccharide is bound, is clearly visible on the left of the structure. Image courtesy of Prof. G. Black, Northumbria University.

for the 3D structure of the enzyme pectate lyase, a virulence factor in some plant pathogenic bacteria.

Using other Web resources

Several websites have been constructed to bring together bioinformatic data and related information for a single organism – many of these sites have been produced as a result of a genome-sequencing project for that particular organism, for example the human genome mapping project. In addition to the nucleic acid and/or protein sequence data available in the primary databases, the organism-specific websites provide a broader range of information and resources, often including links to other Web locations. As such, they can be a useful means of accessing relevant internet sites: Table 70.2 gives examples, along with the locations of several other relevant websites that may be useful when researching a topic, for example as part of an assignment, or when planning a bioinformatics project.

Table 70.2 Other Internet locations relevant to bioinformatics.

Databases and resources for particular organisms	
<i>Arabidopsis</i> information resource	https://www.arabidopsis.org
<i>Drosophila</i> database (flybase)	https://flybase.org
<i>Caenorhabditis</i> genomes project	https://wormbase.org/
<i>E. coli</i> genome project	https://www.genome.wisc.edu
Human genome database	https://www.genome.gov/
HIV sequence database	https://www.hiv.lanl.gov/
Plant genome database	http://www.plantgdb.org/
<i>Saccharomyces</i> genome database	http://www.yeastgenome.org/
SARS-CoV-2 resources	https://www.ncbi.nlm.nih.gov/sars-cov-2/
Other resources	
BioCyc Database Collection (pathways/genomes)	https://biocyc.org
KEGG metabolic pathway database	https://www.genome.jp/kegg/

Sources for further study

(Note also the websites and associated resources, as detailed in Tables 70.1 and 70.2.)

Brown, T.A. (2017) *Genomes 4*. Garland Science, New York.

[This book focuses on genomics, in contrast to more traditional molecular biology textbooks, which emphasise individual genes.]

Buffalo, V. (2015) *Bioinformatics Data Skills*. O'Reilly, Cambridge.

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Korenberg, M.J. (2014) *Microarray Data Analysis*. Humana, New York.

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Ramsden, J. (2015) *Bioinformatics: An Introduction*, 3rd edn. Springer, New York.

Richards, J.E. and Hawley, R.S. (2010) *The Human Genome: a User's Guide*, 3rd edn. Academic Press, London.

STUDY EXERCISES

70.1 Investigate the potential of the Internet for locating information relevant to bioinformatics. Using the Web, answer the following questions:

- How many nucleotide sequences are currently held by GenBank?
- What and where is Hinxton Hall?
- Where is the European Molecular Biology Laboratory (EMBL) Mouse Biology Program located?
- What is the total number of protein structures currently held by the Protein Data Bank?
- What is the full postal address of the US National Center for Biotechnology Information?

70.2 Search GenBank nucleotide database using a DNA sequence. Carry out a BLASTN search of GenBank (at: <http://www.ncbi.nlm.nih.gov/BLAST>) for the following nucleotide sequence: actgactcatagagtaccaccgtggtgct. How many 'hits' do you find? What is the length of the sequence showing complete identity?

70.3 Search the NCBI database using a protein sequence. Carry out a BLASTP search (at: <http://www.ncbi.nlm.nih.gov/BLAST>) for the following amino acid sequence: NVKAAWGKVGHAHAGEYGAE (note – p. 423 gives details of the code letters corresponding to each

amino acid), (a) What protein is this sequence most likely to correspond to? (b) Which sequence gives the highest alignment score? (c) What is the highest score for a sequence from *Homo sapiens*?

70.4 Convert a DNA base sequence to an amino acid sequence. Using the 'Translate' feature of the ExPASy server (at: <https://web.expasy.org/translate/>), carry out a six-frame translation of the following nucleotide sequence: tcagagagaacccaccat. Which of the six frames contains both a start and stop codon and what is the predicted amino acid sequence for this frame?

70.5 Use the Protein Data Bank to investigate the structure of an enzyme. Search the PDB archive (at: <https://www.rcsb.org/pdb/>) using 'beta-galactosidase' as the keyword in the SearchLite search tool, and locate the entry for the *b*-galactosidase (= *b*-glycosidase) enzyme of *Sulfolobus solfataricus*, deposited on 19 September 1996. Use the 'explore' command to find out: (a) the number of subunits in the holoenzyme; (b) the number of amino acids residues in each chain; (c) the number of helices in each chain. Next, use the 'view structure' option to look at the 3D configuration (either in quick PDB format, or using *Rasmol*, if this program is available on your PC, or network): (d) save an image as a file and then incorporate it into a *Word* document, as you might in an assignment.

Answers to these study exercises are available at go.pearson.com/uk/he/resources



Analysing and presenting data

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71 Manipulating and transforming raw data

As part of your lab work or research you may undertake surveys or experiments and produce large amounts of data to be analysed. Exploratory data analysis is the process whereby you refine these data sets and attempt to find meaning within your results. Its primary aims are to:

- **check for missing data, erroneously recorded data and other mistakes**, such as incorrect spelling
- **review the presence of outliers or anomalous data** and investigate the possible reasons for these
- **gain insights about the nature of your results**, for example their scale, range and distribution, including estimates of statistical parameters associated with the data set(s)
- **suggest possible patterns and relationships within the data set**, including identifying influential variables
- **help you to design better surveys or experiments** in future investigations
- **validate the assumptions behind models or hypothesis testing** that you may wish to carry out.

Setting up pilot experiments and surveys – these are preliminary studies carried out to improve technique, gauge the nature of the data that will be obtained and anticipate possible problems in data collection and analysis.

Summarising your results – original, unsummarised data values belong only in your primary record, either in laboratory books or as computer records. You should therefore produce summary tables to organise and condense original data before going any further with analysis or before discussing your results with your tutor.

Assessing outliers – the first thing to look for is an error in recording or transcription of the data, which should be checked by going back to the original record; the second is the possible effects of a confounding variable (p. 207) on the result. For example, was the specimen at the edge of the sampling area which might have given it a different exposure to environmental conditions, or might a pre-existing condition have affected the measured response (such as subject with a pre-existing medical condition)?

KEY POINT Spreadsheets (Chapter 72) are invaluable tools for data manipulation and transformation: complex mathematical procedures can be carried out rapidly and the results visualised almost immediately using the inbuilt graphing functions. Spreadsheets also facilitate the statistical analysis of data (see Chapters 76 and 77).

Carrying out exploratory analysis

To start the process of organising, manipulating and summarising your data, first transcribe or download the values, as appropriate. This should generally result in a table, preferably as a spreadsheet file. Now you can start the process of simplifying and refining the data set:

- **Simplify numbers or categories** – this avoids the detail becoming overwhelming and prevents spurious accuracy (p. 189). If the number-formatting feature is used within a spreadsheet to reduce the number of significant figures (pp. 582–4), you will not lose the inherent values in the data as these will merely be hidden from view. For qualitative data, you may wish to combine categories or simplify the way in which they are described.
- **Identify missing and erroneous values** – here, your aim is to spot data values which may have been incorrectly recorded. These might be revealed through a spell check for qualitative data or as blank values or outliers in a simple chart for quantitative data.
- **Organise data table(s)** – you may wish to add titles for the rows and columns, swap row and columns as appropriate, or create new rows including, for example, mean values (Chapter 76).
- **Display the results in graphical form** – here, your aim is to create an immediate visual summary that both simplifies the data set and

Colour	Tally	Total
Green	III	3
Blue	IIII	4
Red	IIII	4
White	IIIIII	6
Black	I	1
Maroon	III	3
Yellow	II	2
		<hr/> 33

Fig. 71.1 An example of a tally chart.

Producing a histogram – a neatly constructed tally chart will double as a rough histogram, as illustrated by Fig. 71.1.

Table 71.1 An example of a frequency table

Size class	Frequency	Relative frequency (%)
0–4.9	7	2.6
5–9.9	23	8.6
10–14.9	56	20.9
15–19.9	98	36.7
20–24.9	50	18.7
25–29.9	30	11.2
30–34.9	3	1.1
Total	267	99.8*

* \neq 100 due to rounding error.

allows you to spot potential relationships between variables – these are generally easier to detect in graphic form. By rearranging your data in different ways, you might identify patterns and aid interpretation.

- **Move from graphical interpretations to well-chosen numerical summaries and/or verbal descriptions**, including where applicable an explanatory hypothesis.

Visualising your data

The following methods are useful to provide a quick assessment of your results.

Outlining the frequency distribution

In many instances, the first step in analysis is to count how frequently each value occurs and to produce a frequency table. The frequency is simply the number of times a value occurs in the data set, and is, therefore, a count. The raw data could be acquired using a tally chart system to provide a simple frequency table. To construct a tally chart (for example, Fig. 71.1):

- **enter one data value (datum) at a time**, spacing the entries neatly
- **if working from a data list, cross out each item on the list as you enter it on to the tally chart**, to prevent double entries
- **check that all values are crossed out at the end** and that the totals agree.

Convert the data to a formal table when complete (for example, Table 71.1). Because proportions are easier to compare than class totals, the table may contain a column to show the relative frequency of each class. Relative frequency can be expressed in decimal form (as a proportion of 1) or as a percentage (as a proportion of 100).

A frequency table will provide an indication of the most common value(s) in your data set, and if the data are quantitative (Chapter 27), whether the distribution of data is symmetrical or skewed. This information will provide you with an idea of the best methods for carrying out further summarisation and analysis of the results (Chapters 76 and 77) and whether a data transformation might be useful (pp. 549–51).

A visual display of a distribution of values is often useful for variables measured on an interval or ratio scale (p. 188). The distribution of a variable can be displayed by a frequency table for each value or, if the possible values are numerous, groups (classes) of values of the variable. Graphically, there are two main ways of viewing such data:

1. **Histograms** (see p. 82 for details), generally used for large samples.
2. **Stem and leaf plots** (for example, Fig. 71.2), best used for samples of less than 100: these retain the actual values and are faster to draw by hand. The main drawback is the limitation imposed by the choice of stem values since these class boundaries may obscure some features of the distribution.

These displays allow you to look at the overall shape of a distribution and to observe any significant deviations from the theoretical ones. Where necessary, you can use data transformations (pp. 549–51) to investigate any departures from standard distribution patterns such as the normal (Gaussian) distribution or the Poisson distribution.

stem	leaves
7	23
7	55
7	6
7	9
8	000
8	233
8	45555
8	77
8	888899
9	0000111111
9	2333333
9	44555555555
9	66677777
9	88889999
10	00

Fig. 71.2 A simple 'stem and leaf' plot of a data set. The 'stem' shows the common component of each number, while the 'leaves' show the individual components, e.g. the top line in this example represents the numbers 72 and 73.

Using summary values – remember that mean values can mask the presence of outliers or the skewness of the data set.

Using graphs to explore your data

– when drawing preliminary graphs by hand or by manipulating spreadsheet settings, ensure that you:

- **avoid clutter in the graph** – leave out grid lines and try to use the simplest graph possible for your purpose;
- **make the values stand out clearly** – attention should focus on the actual data points, not the labels, scale markings, etc.

Note that these suggestions may contrast with the requirements for constructing a graph for formal data presentation.

Comparing location and dispersion

These technical terms are used to describe the 'centre' of the frequency distribution and the spread of the data and are discussed in more detail in Chapter 76. Both will allow you to summarise the data and obtain estimates of relevant population parameters, assisting in the design of further sampling or experimentation (Chapter 29). They may also help in the selection of statistical tests (Chapter 77), which may depend on whether the spread of data in each sample or treatment is roughly equal, or not.

For quick assessments, a mode (p. 594) can easily be obtained for qualitative data from a frequency distribution as the most common value, while a mean (average) value is easily calculated for quantitative data from the total of all data values divided by the number of values (p. 592). The range (p. 594) provides a simple estimate of quantitative data dispersion, but it is essentially based on outlying data. The relative size of these values can be compared graphically using a bar chart or histogram (p. 82).

Examining the relationships between variables

Scatter diagrams can be an effective way investigate trends and relationships in data. These consist of plots of one variable against another, the aim being to compare the strength of putative relationships between variables.

Figure 71.3 provides an illustration of this approach. In Fig. 71.3(a), the data are relatively widely scattered around an assumed (roughly linear) trend. If you were examining this particular data set, you might wish to check the triangular data point to see whether it is a true outlier or a value that has been incorrectly recorded. In Fig. 71.3(b) the data values are closer to an assumed linear trend. In comparing the two charts, you might assume that variable A had a more direct effect on variable C than variable B, on the basis of the closer relationship revealed by the reduced scatter. These data might prompt you to design an experiment to further test the relationship between variables A and C, under more controlled conditions (Chapter 29).

These examples are simple charts; further examples of graphing data, including different types of plot, are given in Chapter 74.

Transforming your data

Transformations are mathematical functions applied to data values. They are particularly valuable where your results are related to areas and volumes (for example, skin area, body mass).

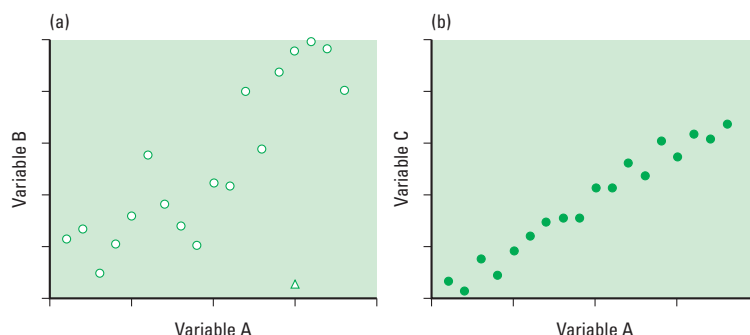


Fig. 71.3 Examples of simple 'scatter' plots being used for data exploration.

Using transformations – note that if you wish to conserve the order of your data, you will need to take negative values when using a reciprocal function (i.e. $-1/n^n$). This is essential when using a box plot to compare graphically the effects of transformations on the five-number summary of a data set.

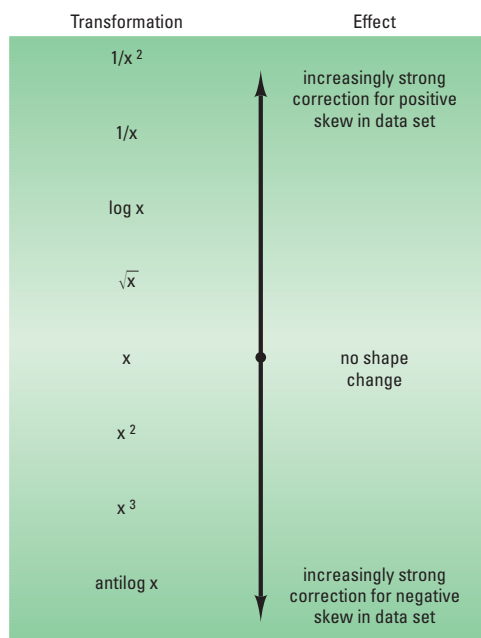


Fig. 71.4 Ladder of transformations (after J.W. Tukey).

The most common use of transformations is to prepare data sets so that specific statistical tests may be applied. For instance, if you find that your data distribution is unimodal but not symmetrical it is often useful to apply a transformation that will redistribute the data values to form a symmetrical distribution. The object of this exercise is often to find the function that most closely changes the data into a standard normal distribution, allowing you to apply a wide range of parametric hypothesis-testing statistics (see Chapter 77). A frequently used transformation is to take logarithms of one or more sets of values: if the data then approximate to a normal distribution, the relationship is termed 'log-normal'.

Some general points about transformations are:

- **they should be made on the raw data, not on derived data values:** this is simpler, mathematically valid, and more easily interpreted
- **the transformed data can be analysed like any other numbers**
- **transformed data can be examined for outliers,** which may be more important if they remain after transformation.

Figure 71.4 presents a ladder of transformations that will help you decide which transformations to try (see also Table 77.1). Note that percentage and proportion data are usually arc-sine transformed, which is a more complex procedure; consult Sokal and Rohlf (2011) for details.

Figure 71.5 illustrates the following 'quick-and-easy' way to choose a transformation:

1. Calculate the 'five-number summary' for the untransformed data (p. 595).
2. Present the summary graphically as a 'box-and-whisker' plot (p. 595).
3. Decide whether you need to correct for positive or negative skew (pp. 597–8).
4. Apply one of the 'mild' transformations in Fig. 71.4 on the five-number summary values only.
5. Draw a new box-and-whisker plot and see whether the skewness has been corrected.

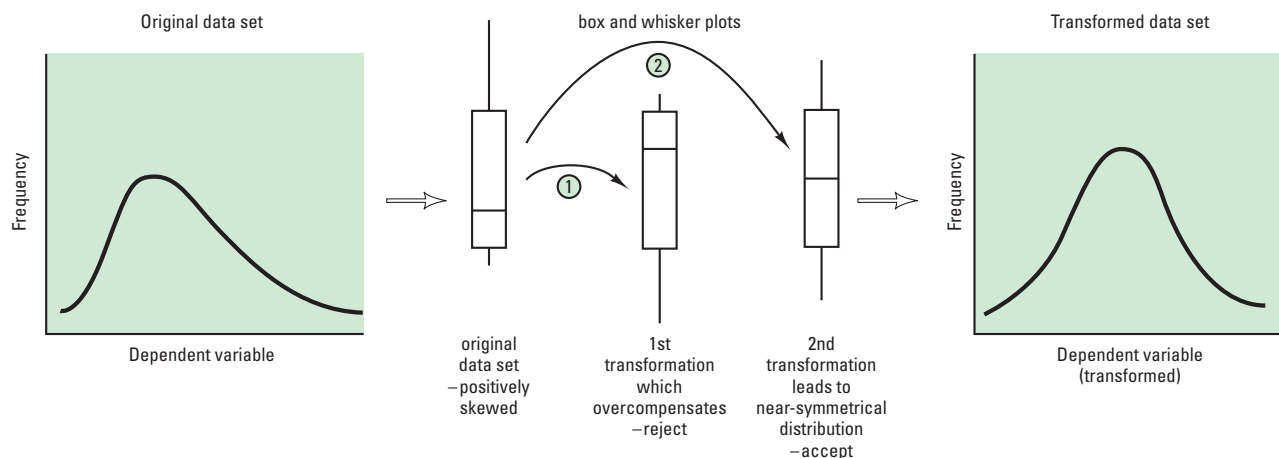


Fig. 71.5 Illustration of the processes of transforming a data set.

6. **If the skewness has not been corrected, try again.** Use a stronger transformation if undercorrected and a milder one if overcorrected.
7. **When the distribution appears to be acceptable, transform the full data set** and recalculate the summary statistics. If necessary, use a statistical test to confirm that the transformed data are normally distributed (pp. 605–7).
8. **If no simple transformation works well, you may need to use non-parametric statistics when comparing data sets.**

Making use of your exploratory analysis

It is likely that your analysis of your initial data set or pilot study will inform later survey or experimental design (Chapter 29). For example, you might:

- **decide to adjust your sampling procedure or the regime for selecting experimental subjects**, perhaps to reduce variability or the possibility of outliers (p. 547)
- **refine the type of survey or experiment** required to best understand the phenomenon under investigation
- **re-assess the amount of data required**, for example, to validate your initial conclusions about a specific difference in characteristics or results, on the basis of estimates of the mean and variance (pp. 595–6)
- **use the preliminary data to select statistical tests**, taking account of the conditions required to satisfy the assumptions of the test methodology
- **choose the best methods for displaying your data in a report** (Chapters 73 and 74).

KEY POINT The main purpose of exploratory analysis is to improve your data collection protocols and help you to refine the methods you use for presenting and analysing your data.

Text reference

Sokal, R.R. and Rohlf, F.J. (2011) *Biometry*, 4th edn. W.H. Freeman, San Francisco.

Sources for further study

Glass, D.J. (2006) *Experimental Design for Biologists*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Quinn, G.P. and Keough, M.J. (2002) *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, Cambridge.

Heath, D. (1995) *An Introduction to Experimental Design and Statistics for Biology*. UCL Press, London.

STUDY EXERCISES

The following study exercises can be most easily carried out using a spreadsheet such as Microsoft *Excel*.

71.1 Compare frequency distributions for samples with different sample sizes. Compute relative frequencies for each class in the table below. Graph the data as a frequency polygon.

Frequency distribution data

Hyphal length (μm)	Treat-ment B	Treat-ment A	Hyphal length (μm)	Treat-ment B	Treat-ment A
0	0	0	13	6	30
1	1	0	14	3	26
2	2	0	15	1	22
3	4	1	16	1	18
4	8	1	17	0	14
5	13	2	18	0	12
6	22	5	19	0	8
7	36	9	20	0	5
8	48	14	21	0	4
9	46	21	22	0	2
10	32	26	23	0	2
11	18	31	24	0	1
12	11	33	25	0	1

71.2 Use a stem and leaf plot. Work out the mean and standard deviation of the data contained in the following stem and leaf plot:

```

6 | 1334
6 | 56788
7 | 013344
7 | 556667899
8 | 11224
8 | 569
9 | 03
9 | 6

```

Stem and leaf plot

71.3 Use transformations to correct skew in a data set. Find a transformation that will make the data of treatment B in Study exercise 71.1 approximately symmetrical about the mean. Demonstrate graphically that you have accomplished this.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

72 Analysing data with spreadsheets

Examples of spreadsheets:

- **Excel** (Microsoft)
- **Numbers** (Apple)
- **Sheets** (Google)
- **Calc** (Apache OpenOffice)

Note: this chapter uses *Microsoft Excel* for illustrative purposes.

Exporting spreadsheet results – data, graphs and tables can be copied and pasted into other compatible programs, such as a word processor in the same office suite.

Table 72.1 Selected examples of spreadsheet formulae and functions. The full range of Excel functions can be found from the *Formulas* menu under *Insert function*

Formula or function	Result
=B4	Copies ('returns') the contents of cell B4 into the current cell.
=B4*B5	Returns the multiplied contents of cells B4 and B5.
=SQRT(B4)	Returns the square root of cell B4). This is an example of a 'math & trig' function (note the abbreviated text form).
=SDEV(A1, B2, C4)	Returns the standard deviation of the three numbers in cells A1, B2 and C4 (note the use of commas to separate the cells). This is an example of a statistical function.
=SUM(B1:D5)	Returns the sum of all the cells in the block bounded by cells B1 and D5 (i.e. the sum of 15 entries). Note the use of the colon to separate the range boundaries. This is an example of a function using a 'range'.
=IF(A1=5, "Five", "Not Five")	Returns the text 'Five' if cell A1 = 5, but if not, returns 'Not Five'. This is an example of a simple logical function.

Spreadsheets are valuable tools in biology for data manipulation and analysis. You can use them for:

- **organising data** – using a spreadsheet as a store for raw data, but also as a simple database, allowing the analysis of text entries
- **automating repetitive calculation** – simplifying these tasks and also reducing the chances of data error due to 'operator fatigue' (Chapter 27)
- **tabulating and graphing data** – producing 'instant' tables and graphic data plots at the point of data entry but also the production of research quality presentations (Chapters 73 and 74)
- **transforming data** – including the application of mathematical functions to data (Chapter 71) as well as the simplification of data into more relevant categories
- **carrying out statistical analysis** – using inbuilt functions to calculate descriptive and hypothesis-testing statistics (Chapters 76 and 77) as well as function-fitting (Chapter 71)
- **predicting from models** – allowing representative data to be entered into a predictive mathematical formula (model) and the output to be compared with real data or results from other formulae.

Being able to take advantage of these analytical approaches requires an understanding of both spreadsheet concepts and the language of relevant commands.

KEY POINT Most spreadsheet programs have broadly similar features. They mainly differ in their user interfaces and the range of inbuilt formulae available.

Understanding spreadsheet concepts

A typical spreadsheet page or 'sheet' (Fig. 72.1) is divided into rows (identified by numbers) and columns (identified by alphabetic characters). Each individual combination of column and row forms a cell that can contain either a data item, a formula or a piece of text. A formula can include scientific and/or statistical functions and/or a reference to other cells or groups of cells (often called a range – examples are shown in Table 72.1). Entry of data into a cell can therefore alter the contents of one or more other cells, meaning that complex systems of data input and analysis can be constructed. New data can be added at any time and the sheet will recalculate automatically, adding the results into any graphics that you have specified.

Spreadsheets have been designed to make assumptions about the nature of data entry being made. If the first character is a number, then the entry is treated as numerical; if it is a letter, then it is treated as a text entry; and if it is a specific symbol ('=' in *Microsoft Excel*), then what follows is a formula. The way you enter a number does not affect the way it is displayed on the screen as this is controlled by cell formatting: this can be adjusted,

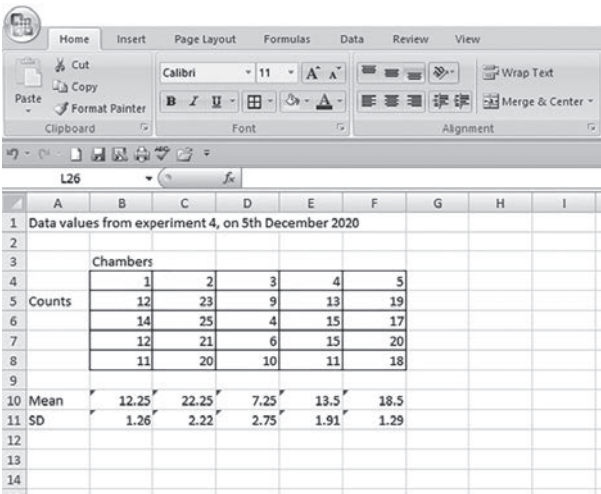


Fig. 72.1 The appearance of typical spreadsheet, showing cells, rows and columns and part of the 'Home' toolbar. This shows an analysis of a simple experiment in *Excel*; the formulae used in rows 10 and 11 are `=AVERAGE(X5:Y8)` and `=SDEV(X5:Y5)` respectively.

(a)			
	Cell	Formula	
Original → cell	A1	=B1+C1	← Original formula
Copied cells ↓	A2	=B2+C2	Copied formulae (relative)
	A3	=B3+C3	
	A4	=B4+C4	

(b)			
	Cell	Formula	
Original → cell	A1	=B1/\$C\$1	← Original formula
Copied cells ↓	A2	=B2/\$C\$1	Copied formulae (mixed relative and absolute)
	A3	=B3/\$C\$1	
	A4	=B4/\$C\$1	

Fig. 72.2 Illustration of relative (a) and absolute (b) copying. In *Excel*, the \$ sign before and after the column letter makes the cell reference absolute, as shown in (b).

Storing set values and constants – it can be useful to place these in a separate area of your spreadsheet, referring to them in formulae using the 'column\$row' convention (e.g. `=$A$3`). This will help you to test the effects of changing them on the spreadsheet output.

for example, to display data with a specific number of significant figures, as a percentage, in currency terms and in scientific notation format for very large and small numbers. If you wish to enter text that starts with a number, then you must type a single quote mark for Microsoft *Excel* (for example, enter '70S ribosome').

All spreadsheets provide a means of copying (replicating) formulae or cell contents when required. This is usually accomplished by 'dragging' a cell's contents to a new range using the mouse. When copying, references to cells may be either *relative*, changing with the row/column as they are copied, or *absolute*, remaining a fixed cell reference and not changing as the formulae are copied (Fig. 72.2). The default for copying is normally relative, so cell references (and, usefully, numbers) will incrementally increase when copied. You can, for instance, drag a formula applying to a specific row down a sequence of rows and the formula will apply to each new row as it did to the first. If you wish a cell reference to be absolute (fixed) when copied, this is done by putting a dollar (\$) sign before and after the column reference letter, for example, `C6`. This is useful when your spreadsheet calculations involve set values or constants.

KEY POINT Understanding the distinction between relative and absolute cell references is very important; it provides one of the most common forms of error when copying formulae.

Setting up a spreadsheet

Layout and design

Spreadsheets can become unwieldy if the basics of layout are not observed, so you should spend some time considering how you would like your spreadsheet to operate – this should include separate areas (effectively tables) for data

Highlighting intermediate calculations – this can be useful as a check on potential errors, in much the same way as you might do this when tackling a numerical problem

entry and for the presentation of results, including graphs. For simple spreadsheets, one solution is to place a summary section at the top of the worksheet and the data section further down. Where the summary and data areas are on the same sheet, you can separate these using ‘panes’ (*View* menu, under *Freeze panes*). This facility allows you to scroll in one part of the sheet (say the data entry section) while still seeing the updated results of data entry in the summary section. Another approach is to separate the ‘results’ or summary section from the raw data by placing them in different sheets, which can be named appropriately. An example of a reference to a cell in another sheet is `=rawdata!G21` – this formula returns the entry in cell G21 in the sheet named ‘rawdata’. To access other sheets, left click on one of the sheet tabs at the bottom of the current spreadsheet and to rename them, right click.

Text labels within cells should always be used to identify parts of your spreadsheet – for example, headers stating what data are contained in a particular column or row, or adjacent text indicating that a cell’s contents represent the end point of a calculation. The latter cells can also be emphasised by the use of bold text, coloured backgrounds and/or borders.

You should control the layout of data in cells, rather than accepting the default settings (see Fig. 72.3). Relevant commands under *Home > Number > Format Cells* include the number of decimal places to be shown or *Scientific* to present data in scientific notation. Note that whatever the type of presentation you choose, the spreadsheet always calculates ‘behind the scenes’ using eight or more places. For presentational purposes, you can also adjust the cell width and the location of the entry within the cell (left, right or centre).

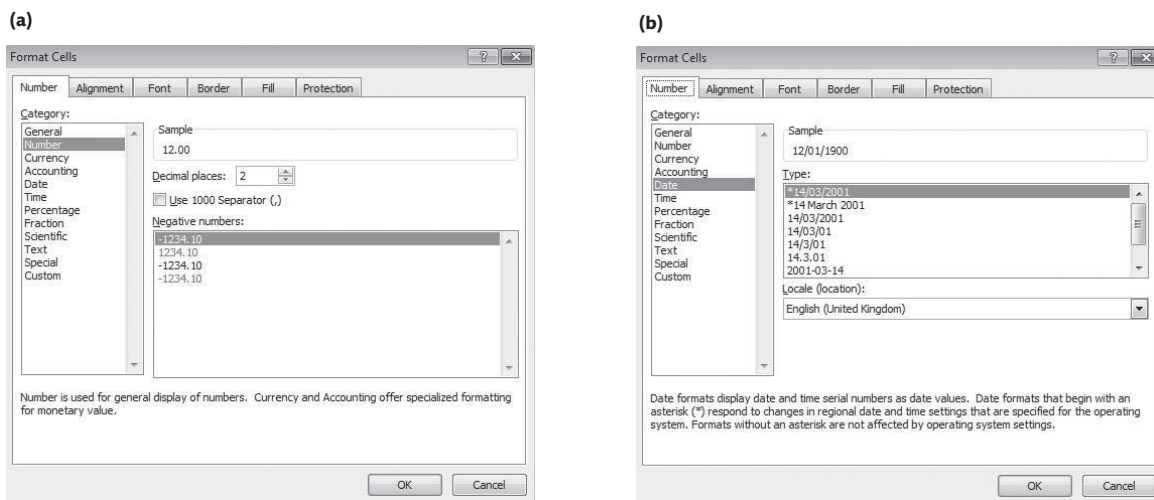


Fig. 72.3 Example of cell-formatting options within the Microsoft *Excel* spreadsheet. These menus are accessed via the *Format > Cell* option and would apply to all of a range of selected cells. (a) Use of the number-formatting option to specify that data will be presented to two decimal places (the underlying data will be held to greater accuracy). (b) Use of the date-formatting option to specify that dates will be presented in day/month/year format. (Spreadsheet dates are stored numerically and converted to appropriate formats. This allows a period between two dates to be calculated more easily.)

Constructing a complex spreadsheet –

start with a simple design and extend it gradually, checking for correct operation as you go. If you do make a mistake in layout, it is a relatively simple matter to copy and paste a section to a new area, but you may need to insert new rows to provide the space to do so.

Importing data output from analytical instruments –

many devices provide output in spreadsheet-compatible form (e.g. as a ‘comma separated values’ (CSV) file). These data sets often include many readings (e.g. from short time intervals) and the spreadsheet can be used to manipulate, analyse and present it according to your needs.

Applying syntax in functions – this is the precise order and formatting of the function, and if not used properly, can result in errors. For example, the syntax for the ‘IF’ function is as follows: IF(logical_test, value_if_true, value_if_false) and an illustration of its use might be =IF(A1>=0, “Positive”, “Negative”). If a comma or set of inverted commas were omitted, then an error message would appear. In some cases, a mistake in syntax could result in an incorrect result, which could be difficult to troubleshoot.

Using logical branches – these can add power to your spreadsheet, for instance by applying a different formula to a result depending on its value.

Data entry and display

At its simplest, data entry consists of typing a number or text into a cell, but you can easily cut and paste information from other sources such as a word processor. Data can also be uploaded from instruments. An auto-entry facility assists greatly when manually entering large amounts of data by moving the entry cursor to the next cell down as data are entered and the return key is pressed. Note that empty cells may be given the value zero by the spreadsheet for certain functions. This may cause errors, for example, by rendering a minimum value inappropriate. Also, an ‘error return’ may result for certain functions if the cell content is zero. Hidden columns are useful for storing intermediate calculations that you do not wish to be displayed on the screen or printout.

Using formulae and functions

These specify the spreadsheet calculations. A cell is referred to by its alphanumeric code, for example, A5 (column A, row 5) and the value contained in that cell manipulated within a formula, for example, = (A5 + 10) or (A5 + B22) in another cell. An example of an *Excel* function is =sin(A5). If you write this in a cell, the spreadsheet will calculate the sine of the number in cell A5 (assuming it to be an angle in radians) and write it in the cell. When a formula refers to another cell or cells, if the value of any of those cells is changed, so is the result of the formula calculation. You can also include branching options in formulae through the use of logical operators (for example, IF, TRUE, FALSE, OR, etc. – for syntax, use the *Formulas > Insert function* menu). Formulae can be ‘nested’ using brackets for more complex calculations.

Spreadsheets work with an order of preference of the standard mathematical operators in much the same way as a standard calculator and this must always be taken into account when operators are used in formulae. The operators ÷ and X are replaced by / and * respectively, while ^ signifies ‘to the power’. In complex formulae, you should use brackets to separate the elements, otherwise the results may not be what you expect. For example, Excel will calculate = A1*B1/C1-D1 differently from (A1*B1)/(C1-D1).

Creating templates

A template is a pre-constructed spreadsheet page containing the formulae required for repeated data analysis. These are recommended whenever you are required to carry out the same calculation task on a routine basis. Most templates start with no entries in the data input cells, and you add your data as results become available. The results are available as soon as the last item is entered. Templates normally contain:

- a data input section
- data transformation and/or calculation sections
- a results section, which can include graphics
- text in the form of headings and annotations
- a summary (output) section.

To create a template, carry out the following sequence of operations:

1. Determine what information/statistics you want to produce.
2. Identify the variables you will need to use, both for original data that will be entered and for any intermediate calculations that might be required.

3. **Set up areas of the template for data entry**, calculation of intermediate values (statistical values such as sums of squares, etc.), calculation of final parameters/statistics and, if necessary, a summary area, where the final results will be displayed.
4. **Decide on the format of the numeric data** if this is different from the default values.
5. **Set the column widths required** for the various headings and data.
6. **Add text (labels) to identify input**, intermediate formulae and output cells. This is valuable in error-tracking and when carrying out further development work.
7. **Enter the formulae required** to make all the calculations, both intermediate and final. Check that results are correct using the test data.
8. **Enter a test set of values** to use during formula entry: use a fully worked example to check that your formulae are working correctly.
9. **Delete all of the test-data values** and you have created your template. Save the template and it is then available for repeated operations.

Printing spreadsheets – this is usually a straightforward procedure, made difficult only by the fact that your spreadsheet may be too big to fit on an A4 sheet of paper. If this could be the case, try to develop a summary area in the sheet that contains only the data that you need to print, or use a specific sheet for the intended output (p. 94). A 'print to fit' option is available in some programs; this forces the output to fit the page dimensions, but for large spreadsheets, this can result in very small text. Always check your layout with the 'print preview' option before printing.

Creating tables and charts for reports and dissertations

Spreadsheets offer a range of graph and chart options, but care must be taken in their use as the default options tend to be business orientated rather than suited to science. In some cases, however, the default options can be suitable for initial data exploration and analysis (for example, Fig. 72.4).

Chapter 74 (Box 74.2) includes illustrations of how to create scientifically acceptable X–Y graphs (*Scatter chart*), bar graphs (*Column chart*), Pie graphs (*Pie chart*) and histograms (*Histogram* function). Note that care should be taken when copying and pasting charts from some versions of Excel to other Microsoft Office products.

Using spreadsheets for modelling

This is where a theoretical prediction or hypothesis is tested, often against 'real' data. A simple example would be to fit a straight line to

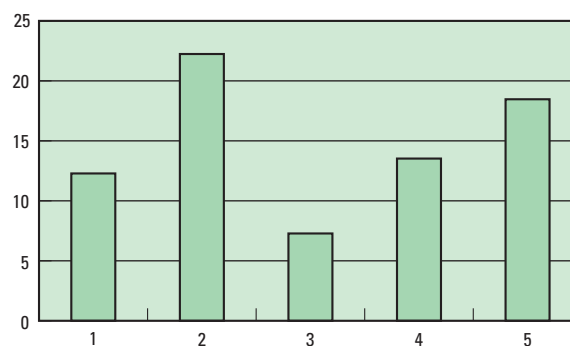


Fig. 72.4 Example of an unmodified Excel chart produced for initial examination of data. This is a 'clustered column' chart of the mean data shown in Fig. 72.1 as created using the '2-D column' option. Note that this graph as shown would *not* be suitable for inclusion in a report, as for example, it lacks axis titles and includes horizontal gridlines (see Chapter 74), but it might be useful to allow preliminary conclusions to be drawn.

a set of data to see whether there appeared to be a direct relationship between two measured variables (see Fig. 77.7). Once the relevant data have been plotted in an X–Y chart, this can be done using the *Chart tools > Layout > Trendline > Linear Trendline* option. The correlation coefficient (r , p. 611) can also be calculated (from *Trendline > More trendline options*) and in this way you might compare different transformations of your data to explore hypothetical relationships between the variables you have plotted – with the r value nearest 1 indicating the best theoretical fit.

This approach can also produce estimated parameters of interest, such as the slope of a linear regression line and the y -intercept. For example, in the ‘classic’ Lineweaver–Burk reciprocal plot for enzyme kinetics (p. 464), you could plot $1/V_{\max}$ against $1/[S]$ to obtain initial estimates of V_{\max} (reciprocal of y -intercept) and K_m (reciprocal of x -intercept) from a straight line fit to your data – but you should be aware of some of the statistical pitfalls in this method.

Using spreadsheets as databases

Formal database programs such as Microsoft *Access* are used to store, filter and analyse large amounts of data, especially information with a high textual content. The computerised catalogue in your library is an example of a database; you normally enter the filter requirements in the form of author or subject keywords. Their construction requires specialist skills, but informal databases that would fit most undergraduate needs can be constructed within spreadsheets, using the columns and rows as ‘fields’ and ‘records’ respectively. The data set can then be analysed using simple

Definitions

Database – a (computerised) filing system which stores related information (‘records’) and allows you to catalogue, search, sort and explore relationships for selected parts of these

File – a collection of records in a database

Field – the individual items of information on each record

Filter – a mechanism for selecting certain files to view

Search criteria – the instructions used to view files through filters, according to your requirements

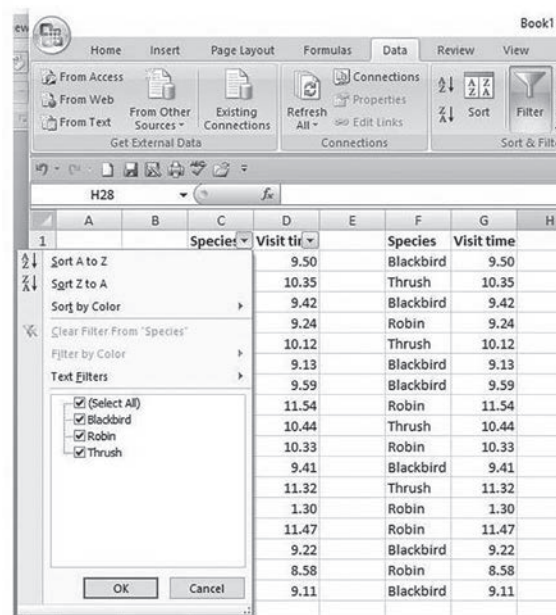


Fig. 72.5 Illustration of a filter being applied to a simple database in *Excel*. Note that the columns of data have been replicated to the right, to allow the full data set to be seen (otherwise obscured by dialog box). Here, from a list of the timings of bird visits, the text filter might be used to select out all the data for blackbirds, for example, by ‘unticking’ the other species.

operations which are probably sufficient for most needs – for example: sort, search, count and filter (Fig. 72.5) – or perhaps a combination of logical commands. However, one area where you may decide to create a personal database is when using a specialised bibliographic program like *EndNote* (Chapter 5).

KEY POINT Use a dedicated (formal) database program only after careful consideration, due to the time and effort involved. Can the task be done within a spreadsheet?

Sources for further study

Harvey, G. (2018) *Excel 2019 for Dummies*. Wiley, New York.
[Editions are available for the other versions of *Excel*.]

Lambert, J. and Frye, C. (2018) *Microsoft Office 2019 Step-by-Step*. Microsoft Press, Redmond.

Lawani, L. (2019) *Excel 2019 All-in-One*. BPB Publications, Noidia.

STUDY EXERCISES

The instructions and tips for these problems assume that you have *Excel* (97 or later) available. If not, they should be readily modified for most advanced spreadsheet programs. If you have problems with any of the tasks, consult Box 74.2 or try using the program's *Help* facility.

72.1 Create a spreadsheet and graph (introductory).

- Copy the information in the table into a spreadsheet. Name and save the spread sheet file appropriately.
- From the copied information, create a pie chart using the *Insert* tab and *Pie* (chart) function.
- Adjust the colours selected so the chart will print out in black and white. Save the final version of

your spreadsheet. Print the chart out directly from *Excel*.

Relative percentages of the major types of leukocytes in a blood sample

Cell type	Percentage
Neutrophils	60
Eosinophils	4
Basophils	1
Monocytes	7
Lymphocytes	28
Total	100

(continued)

STUDY EXERCISES (continued)**72.2 Create a spreadsheet and graph (advanced).**

- (i) Copy the data in the table immediately below into a spreadsheet. Name and save the file appropriately.

Time course of cell population growth

Time (h)	Cell count (per unit volume)
1	2.00
2	6.00
6	50.0
75	6.00×10^3
18	2.20×10^5
24	1.65×10^7

- (ii) Use the spreadsheet and chart-making facilities to explore by eye which of the following transformations would result in the best linear fit

for these data: reciprocal, square root, cube root or log.

- (iii) Add a linear trend-line to the chart for the most appropriate transformation.

- (iv) Copy the graph to a file in *Word* and print out.

72.3 Use a spreadsheet as a simple database.

Copy the data in the table below into cells within a spreadsheet. Modify the column widths so you can see all of the text on a single screen. Now sort the data in the following ways:

- by subject, in alphabetical order;
- by date and then by time of day;
- by topic, in reverse alphabetical order.

Use the *Hide* function (right click on selected column header) so that the information in columns 4 and 6 is not displayed. Find out how to undo this operation.

My exam timetable

Subject	Date	Time	Paper	Location	Question style
Biochemistry	3 Jun	Morning	1	Great Hall	Multiple-choice
Biochemistry	17 Jun	Morning	2	Exam Hall 5	Essay paper
Biochemistry	2 Jun	Afternoon	3	Main Laboratory	Information processing
Ecology	3 Jun	Afternoon	A	Small Hall	Short-answer questions
Ecology	14 Jun	Afternoon	B	Exam Hall 5	Essay paper
Biology A	4 Jun	Morning	1	Small Hall	Short-answer questions
Biology B	1 Jul	Afternoon	1	Exam Hall 3	Short-answer questions
Biology A	13 Jun	Afternoon	2	Exam Hall 5	Essay paper
Biology B	2 Jun	Morning	2	Main Laboratory	Essay paper

Answers to these study exercises are available at go.pearson.com/uk/he/resources

73 Summarising data in tables

A table is often the most appropriate way to present numerical data in a concise, accurate and structured form. Assignments and project reports should contain tables that have been designed to condense and display results in a meaningful way and to aid numerical comparison. The preparation of tables for recording primary data is discussed on p. 215.

Decide whether you need a table, or whether a graph is more appropriate. Histograms and plotted curves can be used to give a visual impression of the relationships within your data. On the other hand, a table gives you the opportunity to make detailed numerical comparisons.

KEY POINT Always remember that the main purpose of your table is to communicate information and allow appropriate comparison, not simply to put down the results on paper.

Preparing the elements of tables

Title

Constructing titles – take care over titles as it is a common mistake in student practical reports to present tables without titles, or to misconstrue the title.

Every table must have a brief descriptive title. If several tables are used, number them consecutively so they can be quoted in your text. The titles within a report should be compared with one another, making sure they are logical and consistent and that they describe accurately the numerical data contained within them.

Structure

Using rulings in tables – modern style is to use horizontal rulings only, so if your word processor or spreadsheet includes vertical lines as default, you may wish to remove these.

Display the components of each table in a way that will help the reader understand your data and grasp the significance of your results. Organise the columns so that each category of like numbers or attributes is listed vertically, while each horizontal row shows a different experimental treatment, organism, sampling site, etc. (as in Table 73.1). Where appropriate, put control values near the beginning of the table. Columns that need to be compared should be set out alongside each other. Use rulings to subdivide your table appropriately, but avoid cluttering it up with too many lines.

Table 73.1 Characteristics of selected photoautotrophic microbes

Division	Species	Optimum [NaCl]* (mol m ⁻³)	Intracellular carbohydrate	
			Identity	Quantity† (nmol (g dry wt) ⁻¹)
Chlorophyta	<i>Scenedesmus quadruplicatum</i>	340	Sucrose	49.7
	<i>Chlorella emersonii</i>	780	Sucrose	102.3
	<i>Dunaliella salina</i>	4700	Glycerol	910.7
Cyanobacteria	<i>Microcystis aeruginosa</i>	<20*	None	0.0
	<i>Anabaena variabilis</i>	320	Sucrose	64.2
	<i>Rivularia atra</i>	380	Trehalose	ND

*Determined after 28-day incubation in modified Von Stosch medium.

†Individual samples, analysed by gas-liquid chromatography.

*Poor growth in all media with added NaCl (minimum NaCl concentration 5 mol m⁻³).

ND Sample lost: no quantitative data.

Examples If you measured the width of a fungal hypha to the nearest one-tenth of a micrometre, quote the value in the form '52.6 μm ', rather than 0.0000526 m or 52.6×10^{-6} m.

Saving further space in tables – in some instances a footnote can be used to replace a whole column of repetitive data.

Using spreadsheets and word-processing packages – these can be used to prepare high-quality versions of tables for project work (Box 73.2).

Headings and subheadings

These should identify each set of data and show the units of measurement, where necessary. Make sure that each column is wide enough for the headings and for the longest data value.

Numerical data

Within the table, do not quote values to more significant figures than necessary, as this will imply spurious accuracy (p. 189). By careful choice of appropriate units for each column you should aim to present numerical data within the range 0 to 1000. As with graphs, it is less ambiguous to use derived SI units, with the appropriate prefixes, in the headings of columns and rows, rather than quoting multiplying factors as powers of 10. Alternatively, include exponents in the main body of the table (see Table 24.1), to avoid any possible confusion regarding the use of negative powers of 10.

Other notations

Avoid using dashes in numerical tables, as their meaning is unclear; enter a zero reading as '0' and use 'NT' not tested or 'ND' if no data value was obtained, with a footnote to explain each abbreviation. Other footnotes, identified by asterisks, superscripts or other symbols in the table, may be used to provide relevant experimental detail (if not given in the text) and an explanation of column headings and individual results, where appropriate. Footnotes should be as condensed as possible. Table 73.1 provides examples.

Statistics

In tables where the dispersion of each data set is shown by an appropriate statistical parameter, you must state whether this is the (sample) standard deviation, the standard error (of the mean) or the 95% confidence limits and you must give the value of n (the number of replicates). Other descriptive statistics should be quoted with similar detail, and hypothesis-testing statistics should be quoted along with the value of P (the probability). Details of any test used should be given in the legend, or in a footnote.

Text

Sometimes a table can be a useful way of presenting textual information in a condensed form (see examples on pp. 501–2).

When you have finished compiling your tabulated data, carefully double-check each numerical entry against the original information, to ensure that the final version of your table is free from transcriptional errors. Box 73.1 gives a checklist for the major elements of constructing a table.

Box 73.1 How to prepare a table

Every table should have the following components:

1. **A title**, plus a reference number and date where necessary.
2. **Headings for each column and row**, with appropriate units of measurement.
3. **Data values**, quoted to the nearest significant figure and with statistical parameters, according to your requirements.
4. **Footnotes** to explain abbreviations, modifications and individual details.
5. **Rulings to emphasise groupings** and distinguish items from each other.

Box 73.2 How to use a word processor or a spreadsheet to create a table for use in coursework reports and dissertations

A. Creating tables with Microsoft Word

Word-processed tables are suitable for text-intensive or number-intensive tables, although in the second case entering data can be laborious. When working in this way, the natural way to proceed is to create the 'shell' of the table, add the data, and then carry out final formatting on the table.

- 1. Move the cursor to the desired position in your document** – this is where you expect the top left corner of your table to appear. Click the *Insert* tab, then choose *Table*.
- 2. Select the appropriate number of columns and rows** – do not forget to add rows and columns for headings. As default, a full-width table will appear, with single rulings for all cell boundaries, with all columns of equal width and all rows of equal height.

Example of a 4 × 3 table:

- 3. Customise the columns** – by placing the cursor over the vertical rulings then 'dragging', you can adjust their width to suit your heading text entries, which should now be added.

Heading 1	Heading 2	Heading 3	Heading 4

- 4. Work through the table adding the data** – entries can be numbers or text.

Heading 1	Heading 2	Heading 3	Heading 4
Xx	xx	xx	xx
Xx	xx	xx	

- 5. Make further adjustments to column and row widths to suit** – for example, if text fills several rows within a cell, consider increasing the column width, and if a column contains only single or double-digit numbers, consider shrinking its width. To combine cells, first highlight them, then right-click the mouse button (or equivalent) and click on *Merge Cells*. You may wish to reposition text

within a cell by right-clicking the mouse button and choosing an appropriate position in the cell alignment menu.

Heading 1	Heading 2	Heading 3	Heading 4
Xx	Xx	xx	xx
	xx	xx	

- 6. Finally, remove selected borders to cells** – one way is to highlight the table, then click on the *Design* tab and choose an appropriate style from the template options. If you do not want any shading, click on *Design* > *Shading* and choose *No color*. Another way is to highlight the cells in the table, then right-click the mouse button and choose *Borders and Shading*. You can choose the style you wish from this submenu so that your table looks like the examples shown in this chapter.
- 7. Add a table title** – this should be positioned above the table (cf. a figure title and legend, p. 566), legend and footnotes.

Final version of the table:

Table xx. A table of some data

Heading 1	Heading 2 ^a	Heading 3	Heading 4
Xx	xx	xx	xx
	xx	xx	xx

^aAn example of a footnote.

B. Creating tables with Microsoft Excel

Tables derived from spreadsheets are effective when you have lots of numerical data, especially when these are stored or created using the spreadsheet itself. When working in this way, you can design the table as part of an output or summary section of the spreadsheet, add explanatory headings, format, then possibly export to a word processor when complete.

- 1. Design the output or summary section.** Plan this as if it were a table, including adding text headings within cells.

16				
17				
18	Heading 1	Heading 2	Heading 3	Heading 4
19				
20				
21				
22				

(continued)

Box 73.2 (continued)

- 2. Insert appropriate formulae within cells to produce data.** If necessary, formulae should draw on the other parts of the spreadsheet.

17				
18	Heading 1	Heading 2	Heading 3	Heading 4
19	Aaa	=A1	=C3*5	=SDEV(A1:A12)
20	Bbb	=A2	=F45/G12	=SDEV(B1:B12)
21				
22				

- 3. Format the cells.** This is important to control the number of decimal places presented (*Home* > *Number* > *Format* > *Cells*).
- 4. Adjust column width to suit.** You can do this via the column headings, by placing the cursor over the rulings between columns then 'dragging'.

17				
18	Heading 1	Heading 2	Heading 3	Heading 4
19	Aaa	=A1	=C3*5	=SDEV(A1:A12)
20	Bbb	=A2	=F45/G12	=SDEV(B1:B12)
21				
22				

- 5. Add rulings as appropriate.** One way is to use the *Home* > *Font* > *Borders* menu, having selected the relevant cells.

17				
18	Heading 1	Heading 2	Heading 3	Heading 4
19	Aaa	=A1	=C3*5	=SDEV(A1:A12)
20	Bbb	=A2	=F45/G12	=SDEV(B1:B12)
21				
22				

- 6. Add 'real' data values to the spreadsheet.** This should result in the summary values within the table being filled. Check that these are presented with the appropriate number of significant figures (pp. 582–4).
- 7. The table can now be copied and pasted to a Word document.** For best results, use the *Paste Special...* > *Microsoft Office Excel Worksheet Object* option.

Note: instructions and commands illustrated here may vary among the various versions of Microsoft *Office* programs and for different products.

Sources for further study

Kirkup, L. (2019) *Experimental Methods: An Introduction to the Analysis and Presentation of Data*, 2nd edn. Cambridge University Press, Cambridge.

New York State University. *Labwrite Resources: Graphing Resources*. Available: <https://labwrite.ncsu.edu/res/res-homepage.htm>
Last accessed 17/05/21. [Useful resources on both tables and their links to graphics.]

Willis, J. (2004) *Data Analysis and Presentation Skills: An Introduction for the Life and Medical Sciences*. Wiley, Chichester.

STUDY EXERCISES

73.1 Redesign a table of data. Using the following example, redraft the table to improve layout and correct inconsistencies.

Concentrations of low molecular weight solutes in bacteria

Bacterium	Constituent	Concentration
<i>Escherichia coli</i>	Proline	21.0 mmol L ⁻¹
<i>Escherichia coli</i>	Trehalose	1.547 × 10 ² kmol m ⁻³
<i>Bacillus subtilis</i>	Proline	39.7 mmol L ⁻¹
<i>Bacillus subtilis</i>	Glutamate	0.0521 mmol cm ⁻³
<i>Staphylococcus aureus</i> *	Glutamate	15 260 mmol m ⁻³
<i>Escherichia coli</i>	Glutamate	0.50% w/v
<i>Bacillus subtilis</i>	Trehalose	<0.001% w/v

*Proline and trehalose were not measured.

73.2 Devise a text-based table. After reading through this chapter and working from memory, draw up a table listing the principal components of a typical table in the

first column, and brief comments on the major features of each component in the second column.

73.3 Interpret data from a table. The table below shows representative data for energy, protein and niacin (vitamin B3) as a function of age for human males and females. The typical average mass ('weight') of each class is also shown.

- In what age class does the mass of females exceed that of males?
- In what age class is the energy requirement of males greatest in proportion to the energy requirement of females?
- Over what age range do males and females have the same protein requirement?
- In what age class do males have their highest niacin requirement, and how does this compare with females?

Selected age-related characteristics of human males and females

Age (years)	Average mass (kg)		Energy requirement (MJ)		Protein requirement (g)		Niacin requirement (mg)	
	Male	Female	Male	Female	Male	Female	Male	Female
<1	8	8	3.4	3.2	14	14	6	6
1-3	13	13	5.2	4.9	16	16	9	9
4-6	20	20	7.2	6.5	24	24	12	12
7-10	28	28	8.2	7.3	28	28	13	13
11-14	45	46	9.3	7.7	45	46	17	15
15-18	66	55	11.5	8.8	59	44	20	15
19-50	79	63	10.6	8.1	63	50	19	15
>50	77	65	9.5	7.7	63	50	15	13

Answers to these study exercises are available at go.pearson.com/uk/he/resources

74 Illustrating data in graphs

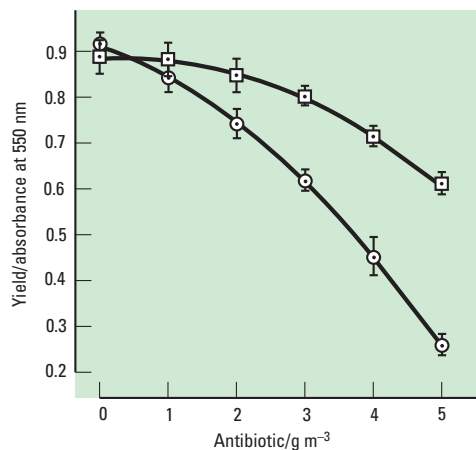
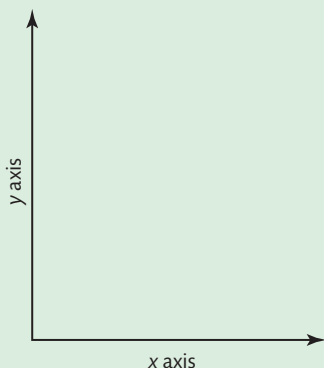


Fig. 74.1 Effect of antibiotic on yield of two bacterial isolates: ○, sensitive isolate; □, resistant isolate. Vertical bars show standard errors ($n = 6$).

Modifying default graphics settings – as well as layout and formatting issues (Box 74.2) you may find that fonts in labels and legends are inconsistent with other parts of your work and that some programs cannot reproduce symbols such as μ (do not use 'u' as a substitute). The same applies to scientific notation and superscripts: do not use 14C for ^{14}C , and replace, for example, $1.4\text{E} + 09$ with 1.4×10^9 . First try cutting and pasting the 'correct' version from Word or, if this fails, leave space and draw the correct symbols by hand on the printout.

Remembering which axis is which – a way of remembering the orientation of the x axis is that x is a 'cross', and it runs 'across' the page (horizontal axis) while y is the first letter of yacht, with a large vertical mast (vertical axis).



Graphs can be used to show detailed results in an abbreviated form, displaying the maximum amount of information in the minimum space. Graphs and tables present findings in different ways. A graph (figure) gives a visual impression of the content and meaning of your results, while a table provides an accurate numerical record of data values. You must decide whether a graph should be used, for example, to illustrate a pronounced trend or relationship, or whether a table (Chapter 73) is more appropriate.

A well-constructed graph will combine simplicity, accuracy and clarity. Planning of graphs is needed at the earliest stage in any write-up as your accompanying text will need to be structured so that each graph delivers the appropriate message. Therefore, it is best to decide on the final form for each of your graphs before you write your text. The text, diagrams, graphs and tables in a laboratory write-up or project report should be complementary, each contributing to the overall message. In a formal scientific communication it is rarely necessary to repeat the same data in more than one place (for example, as a table and as a graph). However, graphical representation of data collected earlier in tabular format may be applicable in laboratory practical reports.

Drawing graphs – practical aspects

The following comments apply to hand-drawn graphs for laboratory reports, project work and dissertations. The same principles also apply to graphs produced using Microsoft *Office* (or open source products) and more specialist data analysis and graphics programs. The difficulty with some programs is that their default graph templates are not suited to science. As a result, you may need to carry out quite extensive modifications to achieve suitable output.

When designing a graph, follow this procedure:

- **Consider the layout and scale of the axes carefully.** Most graphs are used to illustrate the relationship between two variables (x and y) and have two axes at right angles (for example, Fig. 74.1). The horizontal axis is known as the abscissa (x axis) and the vertical axis as the ordinate (y axis).
- **Choose the axis assigned to each variable carefully.** Usually the x axis is used for the independent variable (for example, treatment) while the dependent variable (for example, biological response) is plotted on the y axis (p. 332). When neither variable is determined by the other, or where the variables are interdependent, the axes may be plotted either way round.
- **Create descriptive labels for each axis showing what is represented,** together with the appropriate units of measurement, separated from the descriptive label by a solidus or 'slash' (/), as in Fig. 74.1, or by brackets, as in Fig. 74.2.
- **Add a scale with reference marks ('tics') to each axis** to show clearly the location of all numbers used.
- **Add a figure legend to provide explanatory detail,** including a key to the symbols used for each data set.

Selecting a title – it is a common fault to use titles that are grammatically incorrect: a widely applicable format is to state the relationship between the dependent and independent variables within the title, e.g. ‘The relationship between enzyme activity and external pH’.

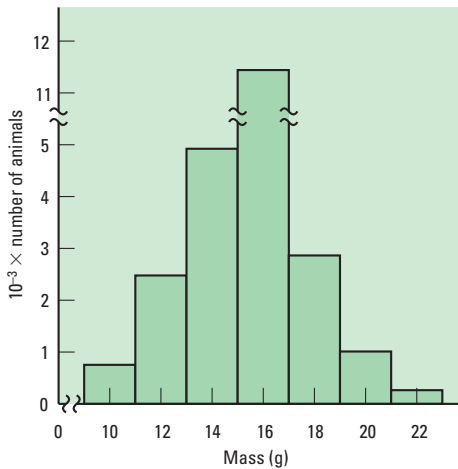


Fig. 74.2 Frequency distribution of masses for a sample of animals (sample size 24 085); the size class interval is 2 g.

Example For a data set where the smallest number on the log axis is 10 and the largest number is 9000, three-cycle log-linear paper would be used, covering the range 10–10 000 (Fig. 74.3).

KEY POINT Graphs should be self-contained – they should include all material necessary to convey the appropriate message without reference to the text. Every graph must have a concise explanatory title to establish the content. If several graphs are used, they should be numbered, so they can be quoted in the text.

Handling very large or very small numbers

To simplify presentation when your experimental data consist of either very large or very small numbers, the plotted values may be the measured numbers multiplied by a power of 10: this multiplying power should be written immediately before the descriptive label on the appropriate axis (as in Fig. 74.2). However, it is often better to modify the primary unit with an appropriate prefix (p. 192) to avoid any confusion regarding negative powers of 10.

Deciding on dimensions

Remember that the purpose of your graph is to communicate information. It must not be too small, so use at least half an A4 page and design your axes and labels to fill the available space without overcrowding any adjacent text. If using graph paper, remember that the white space around the grid is usually too small for effective labelling. The shape of a graph is determined by your choice of scale for the x and y axes which, in turn, is governed by your experimental data. It may be inappropriate to start the axes at zero (for example, Fig. 74.1). In such instances, it is particularly important to show the scale clearly, with scale breaks where necessary, so the graph does not mislead. Note that Fig. 74.1 is drawn with ‘floating axes’ (i.e. the x and y axes do not meet in the lower left-hand corner), while Fig. 74.2 has clear scale breaks on both x and y axes.

Using graph paper

In addition to conventional linear (squared) graph paper, you may be asked to use the following:

- **Probability graph paper.** This is useful when one axis is a probability scale (for example, Fig. 77.5).
- **Log-linear graph paper.** This is appropriate when one of the scales shows a logarithmic progression, for example, the exponential growth of cells in liquid culture (Fig. 34.3). Log-linear paper is defined by the number of logarithmic divisions (usually termed ‘cycles’) covered (for example, Fig. 74.3), so make sure you use a paper with the appropriate

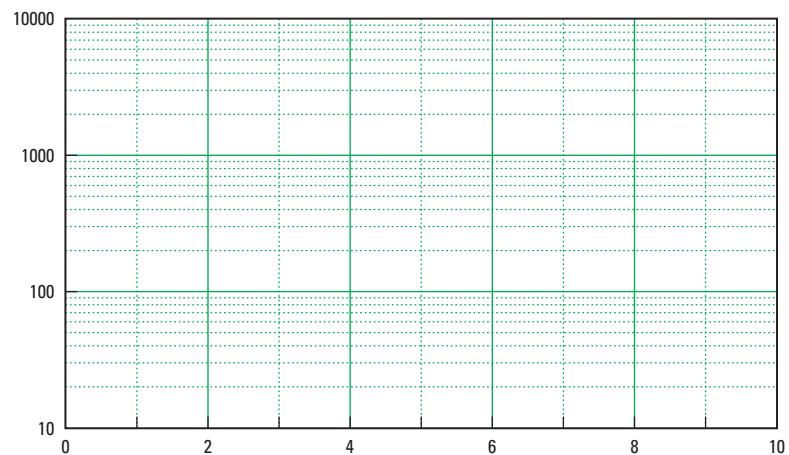


Fig. 74.3 Representation of three-cycle log-linear graph paper, marked up to show a y axis (log) scale from 10 to 10000 and an x axis (linear) scale from 0 to 10.

number of cycles for your data. An alternative approach is to plot the log-transformed values on 'normal' graph paper.

- **Log-log graph paper.** This is appropriate when both scales show a logarithmic progression.

Creating different forms of graph

Different graphical forms may be used for different purposes, including:

- **Plotted curves** are used for data where the relationship between two variables can be represented as a continuum (for example, Fig. 74.4).
- **Scatter diagrams** are used to visualise the relationship between individual data values for two interdependent variables (for example, Fig. 74.5) often as a preliminary part of a correlation analysis (p. 611).
- **Three-dimensional graphs** show the interrelationships of three variables, often one dependent and two independent (for example, Fig. 74.6). A contour diagram is an alternative method of representing such data.
- **Histograms** represent frequency distributions of continuous variables (for example, Fig. 74.7). An alternative is the tally chart (p. 548).
- **Frequency polygons** emphasise the form of a frequency distribution by joining the coordinates with straight lines, in contrast to a histogram. This is particularly useful when plotting two or more frequency distributions on the same graph (for example, Fig. 74.8).
- **Bar charts** represent frequency distributions of a discrete qualitative or quantitative variable (for example, Fig. 74.9). An alternative representation is the line chart (Fig. 77.3).
- **Pie charts** illustrate portions of a whole (for example, Fig. 74.10).
- **Pictographs** give a pictorial representation of data (for example, Fig. 74.11).

Choosing between a histogram and a bar chart – use a histogram for *continuous* quantitative variables and a bar chart for *discrete* variables (see Chapter 27 for details of these types of measurement scale).

The plotted curve

This is the most common form of graphical representation used in biology. The key features are outlined below and given in checklist form in Box 74.1, while Box 74.2 gives advice on using Microsoft *Excel* to create a graph of this type.

Box 74.1 How to approach drawing a graph

The following sequence can be used whenever you need to construct a plotted curve: it will need to be modified for other types of graph.

1. **Collect all of the data values and statistical values** (in tabular form, where appropriate).
2. **Decide on the most suitable form of presentation:** this may include transformation (pp. 549–51) to convert data to linear form.
3. **Choose a concise descriptive title**, together with a reference (figure) number and date, where necessary.
4. **Determine which variable is to be plotted on the x axis and which on the y axis.**
5. **Select appropriate scales for both axes** and make sure that the numbers and their location (scale marks) are clearly shown, together with any scale breaks.
6. **Decide on appropriate descriptive labels for both axes**, with SI units of measurement, where appropriate.
7. **Choose the symbols for each set of data points** and decide on the best means of representation for statistical values.
8. **Plot the points** to show the coordinates of each value with appropriate symbols.
9. **Draw a trend line for each set of points.** Use a see-through ruler, so you can draw the line to have an equal number of points on either side of it.
10. **Write a figure legend**, to include a key that identifies all symbols and statistical values and any descriptive footnotes.

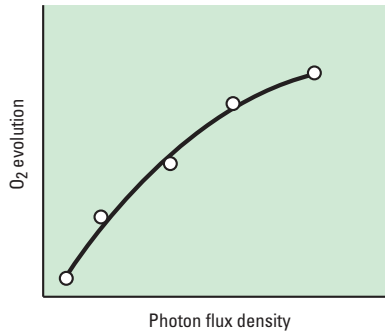


Fig. 74.4 Plotted curve: the rate of photosynthetic O₂ evolution as a function of photon flux density.

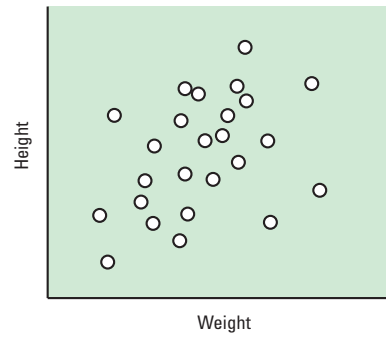


Fig. 74.5 Scatter diagram: height and weight of individual animals in a sample.

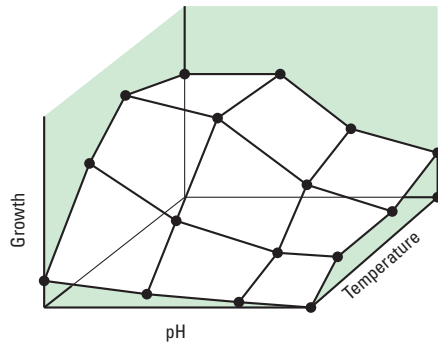


Fig. 74.6 Three-dimensional graph: growth of an organism as a function of temperature and pH.

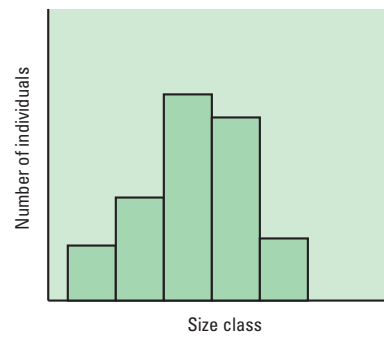


Fig. 74.7 Histogram: the number of plants within different size classes.

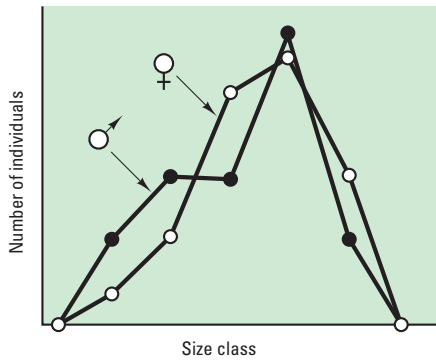


Fig. 74.8 Frequency polygon: frequency distributions of male and female animals according to size.

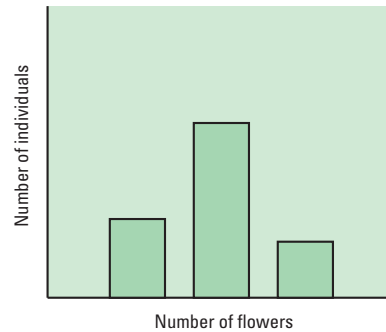


Fig. 74.9 Bar chart: number of flowers per plant.

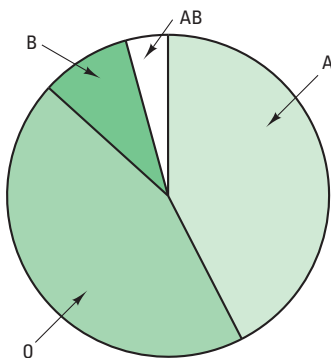


Fig. 74.10 Pie chart: relative abundance of human blood groups.

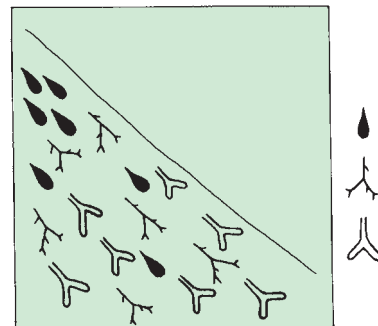


Fig. 74.11 Pictograph: distribution of plants on a rocky shore.

Choosing graphical symbols – plotted curves are usually drawn using a standard set of symbols: ●, ○, ■, □, ▲, △, ◆, ◇. By convention, paired symbols ('closed' and 'open') are often used to represent 'plus' (treatment) and 'minus' (control) treatments.

Adding error bars to Microsoft Excel graphs – you can do this as follows:

1. On your graph, right-click on one of the data points of the series to which you want to add error bars.
2. From *Layout > Analysis > Error Bars* menu select the appropriate option. Typically, you will wish to select *More Error Bars Options > Custom* and chose appropriate options from that menu. You may need to select a data array into which you have entered calculate values for the upper and lower error limits for your chosen statistic over the range of the plotted values. You can adjust the format of the error bars from the *More Error Bars Options* menu.

Note that the commands illustrated above may vary among software versions.

Conveying the correct message – the golden rule is: 'always draw the simplest line that fits the data reasonably well and is biologically reasonable'.

Extrapolating plotted curves – try to avoid the need to extrapolate by better experimental design.

Data points

Each data point must be shown accurately, so that any reader can determine the exact values of x and y . In addition, the results of each treatment must be readily identifiable. A useful technique is to use a dot for each data point, surrounded by a hollow symbol for each treatment (see Fig. 74.1). An alternative is to use symbols only (Fig. 74.4), though the coordinates of each point are then defined less accurately. Use the same symbol for the same entity if it occurs in several graphs and provide a key to all symbols.

Statistical measures

If you are plotting average values for several replicates and if you have the necessary statistical knowledge, you can calculate the standard error (pp. 596–7), or the 95% confidence limits (pp. 610–11) for each mean value and show these on your graph as a series of vertical bars (see Fig. 74.1). Make it clear in the legend whether the bars refer to standard errors or 95% confidence limits and quote the value of n (the number of replicates per data point). Another approach is to add a least significant difference bar (p. 606) to the graph.

Interpolation

Once you have plotted each point, you must decide whether to link them by straight lines or a smoothed curve. Each of these techniques conveys a different message to your reader. Joining the points by straight lines may seem the simplest option, but may give the impression that errors are very low or non-existent and that the relationship between the variables is complex. Joining points by straight lines is appropriate in certain graphs involving time sequences (for example, counts of individuals vaccinated each day), or for repeat measurements where measurement error can be assumed to be minimal (for example, recording a patient's temperature in a hospital, to emphasise any variation from one time point to the next). However, in most plotted curves the best straight line or curved line should be drawn (according to appropriate mathematical or statistical models, or by eye), to highlight the relationship between the variables – after all, your choice of a plotted curve implies that such a relationship exists. Do not worry if some of your points do not lie on the line: this is caused by errors of measurement and by biological variation. Most curves drawn by eye should have an equal number of points lying on either side of the line. You may be guided by 95% confidence limits, in which case your curve should pass within these limits wherever possible.

Curved lines can be drawn using a flexible curve, a set of French curves, or freehand. In the latter case, turn your paper so that you can draw the curve in a single, sweeping stroke by a pivoting movement at the elbow (for larger curves) or wrist (for smaller ones). Do not try to force your hand to make complex, unnatural movements, as the resulting line will not be smooth. Graphs created in spreadsheets can incorporate a 'best fit' curved line if the right options are selected.

Extrapolation

Be wary of extrapolation beyond the upper or lower limit of your measured values. This is rarely justifiable and may lead to serious errors. Whenever extrapolation is used, a dotted line ensures that the reader is aware of the uncertainty involved. Any assumptions behind an extrapolated curve should also be stated clearly in your text.

Box 74.2 How to create and amend graphs within a spreadsheet for use in coursework, theses and dissertations

Spreadsheets can be used to create graphs of reasonable quality, as long as you know how to amend the default settings so that your graph meets the formal standards required for practical and project reports. As with a hand-drawn graph, the basic stages in graph drawing (Box 74.1) still apply. The following instructions explain how to produce an X-Y graph (plotted curve, p. 568), bar graph (pp. 572–3), pie graph and histogram using Microsoft *Excel*, where all types of graphs are termed 'charts'. Note that the commands illustrated above may vary among software versions.

Producing an X-Y graph (Scatter chart in Excel)

1. Create the appropriate type of graph for your data. Enter the numeric values for your X variable data in the cells of a single column and the equivalent values for the Y variable in the adjacent cells of the next column to the right. Then select the whole data array (highlight the appropriate cells by clicking and holding down the left mouse button and dragging the cursor across the cells so that all values are included). Then select the *Insert* tab at the top of the sheet, and select (left-click) the *Scatter* chart from the options provided in the upper ribbon. Note that you should never use the *Line* chart option, as it is based on an x axis that is not quantitative, so all X values will appear as equally spaced categories, rather than having a true scale). Select the first option from the *Scatter* menu (*Scatter with only Markers*). Once selected, this will produce an embedded scatter chart of the type shown in Fig. 74.12(a). The line is then added later, as described below.
2. **Change the default settings to improve the appearance of your graph.** Consider each element of the image in turn, including the overall size, height and width of the graph (resize by clicking and dragging one of the 'sizing handles' around the edge of the chart). The graph shown in Fig. 74.12(b) was produced by altering the default settings, typically by moving the cursor over the feature and then clicking the right mouse button to reveal an additional menu of editing and formatting options. (Note that the example given below is for illustrative purposes only, and should not necessarily be regarded as prescriptive.)

Example for an X-Y graph (compare Fig. 74.12(a) with Fig. 74.12(b)):

- Unnecessary legend box on right-hand side can be removed using the (right-click) *Delete* option.
- Border to chart can be removed using *Format Chart Area* function (available by right-clicking within chart area).
- Gridlines can either be removed, using the *Delete* function or, if desired (as in Fig. 74.12(b)), changed by clicking on

each axis and using the *Add Minor Gridlines* and *Format Gridlines* options to alter the *Colour* and *Style* of the gridlines to make them more like those of conventional graph paper.

- x and y axes can be reformatted by selecting each in turn, and using the *Format Axis* menu options to select appropriate scales for major and minor units, style of tick marks, etc. Remember that it is better to use a figure legend in *Word* (Chapter 14), rather than the *Chart Title* option within *Excel*.
- x and y axis labels can be added by selecting the *Layout* tab, then *Axis Titles*, then *Primary Horizontal Axis Title* and *Primary Vertical Axis Title* options, which will produce a text box beside each axis into which can be typed the axis label and any corresponding units. This can be then changed from the default font using the *Home* tab options.

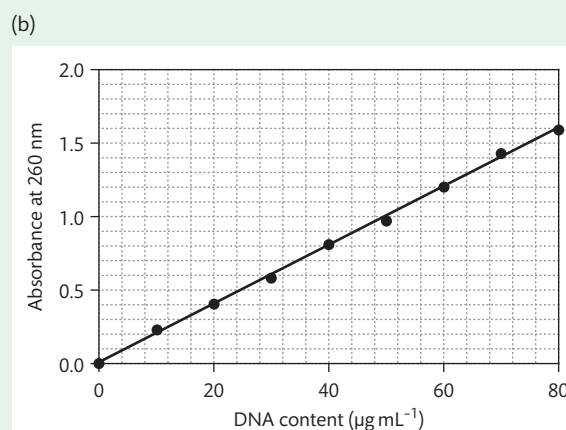
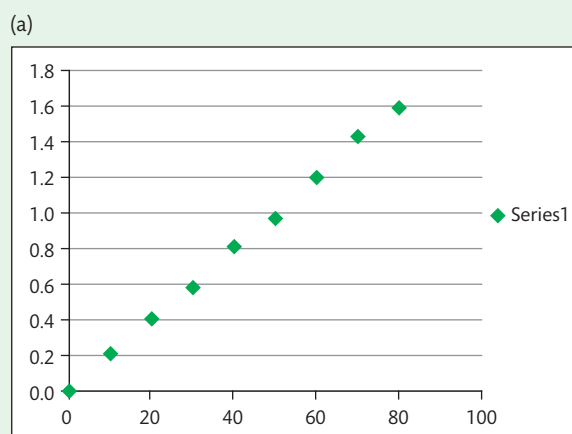


Fig. 74.12 Examples of a plotted curve produced in Microsoft *Excel* using (a) default settings and (b) modified (improved) settings.

(continued)

Box 74.2 (continued)

- Data point style can be changed by selecting (right-clicking) any data point and following the *Format Data Series* options to choose appropriate styles, colours and fill of the data markers.
- A straight line of best fit can be added by selecting any data point and using the *Add Trendline* option to choose a *Linear* line type with appropriate colour and style (explore other options within the *Format*, *Layout* and *Design* tabs at the top of the worksheet).

Producing a bar graph (Column chart in Excel)

1. Create the appropriate type of graph for your data. Enter the category names (for X axis) in one column and the numeric values (for Y axis) in the next column. Select (highlight) the data array, then select the *Insert* tab, and choose the *Column* chart from the options provided. For a standard bar graph, select the first option from the *Column* menu (*Clustered Column*). Once selected, this will produce an embedded bar graph of the type shown in Fig. 74.13(a).
2. **Change the default settings to improve the appearance of your graph.** The bar graph shown in Fig. 74.13(b) was produced by selecting each feature and altering the default settings, as detailed below (illustrative example).

Example for a bar graph (compare Fig. 74.13(a) with Fig. 74.13(b)):

- Unnecessary legend box on right-hand side can be removed using the *Delete* option.
- Border to chart can be removed using *Format Chart Area* function available by right-clicking within chart area).
- Gridlines can either be removed, using the *Delete* function or changed by selecting the gridlines and using the *Format Gridlines* option to alter the *Colour* and *Style*.
- The y axis can be reformatted by selecting the axis, then using the *Format Axis* menu options to select appropriate scales, tick marks, line colour, etc. Note that the x axis should already contain category labels from the spreadsheet cells (modify original cells to update spreadsheet, if necessary).
- x and y axis labels can be added by selecting the *Layout* tab, then *Axis Titles*, then *Primary Horizontal Axis Title* and *Primary Vertical Axis Title* options, as detailed for the plotted curve (Fig. 74.12).
- Bar colour can be modified using the *Format Data Series* menu, selecting appropriate *Fill* and *Border* colours, e.g. white and black respectively in Fig. 74.13(b).
- Individual Y data values can be shown using the *Add Data Labels* option (other options and adjustments can

be made using the *Format*, *Layout* and *Design* tabs at the top of the worksheet).

Note that for all types of graph, it is better not to use the *Chart Title* option within *Excel*, which places the title at the top of the chart (as in Fig. 74.13(a)), but to copy and paste your untitled graph into a word-processed document, such as a Microsoft *Word* file (p. 574 gives details of the procedure), and then type a formal figure legend below the graph, as in Fig. 74.12(b) and Fig. 74.13(b). However, once your graph is embedded into a *Word* file, it is generally best not to make further amendments – you should go back to the original *Excel* file, make the required changes and then repeat the copy-paste procedure to reinsert the graph into the *Word* file.

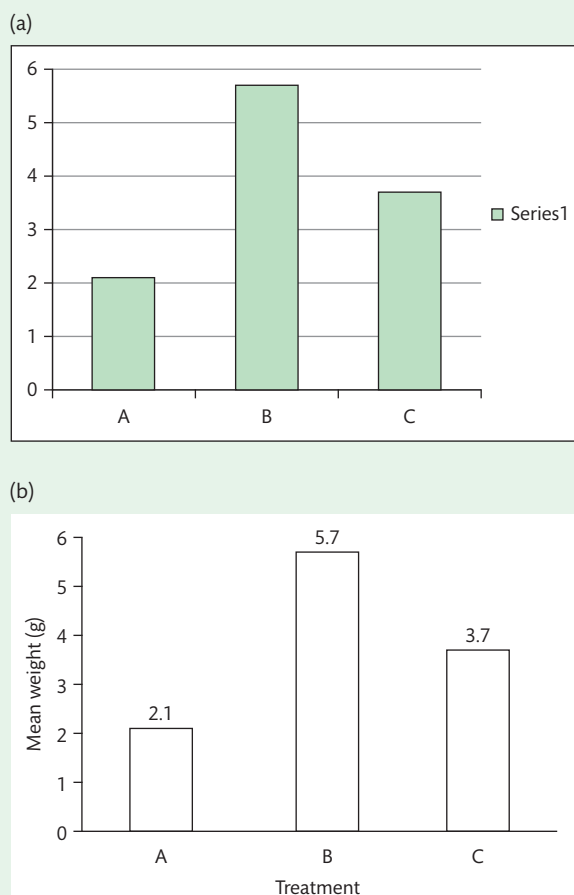


Fig. 74.13 Examples of a bar graph produced in Microsoft *Excel* using (a) default settings and (b) modified (improved) settings.

(continued)

Box 74.2 (continued)**Producing a pie graph (Pie chart in Excel)**

1. Create the appropriate type of graph for your data. Enter the category names for each part of the pie chart in one column, and the corresponding numbers (counts, fractions or percentages) in the next column. Select (highlight) the data array, then select (left-click) the *Insert* tab, and choose the *Pie* chart from the options provided. For a standard pie graph, select the first option from the menu (*Pie*). Once selected, this will produce an embedded pie graph.
2. **Change the default settings to improve the appearance of your graph.** For example, you can show the data values (*Data labels*), adjust colours and shading (e.g. switch from multi-colour to shades of grey), remove chart border, etc., as required (an illustrative example is shown as Fig. 74.14).

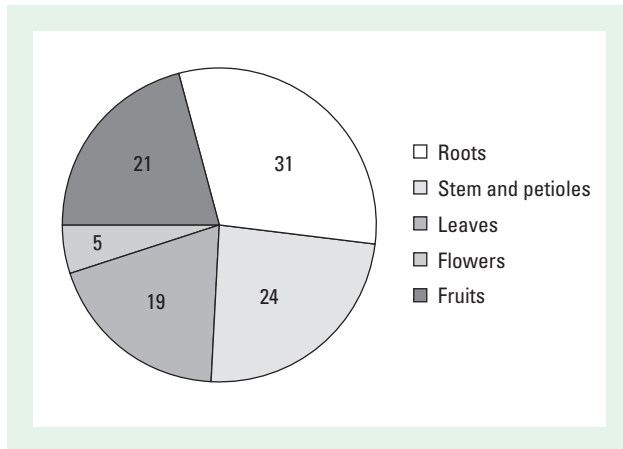


Fig. 74.14 Example of a pie graph produced in Microsoft Excel using format modified from the default settings.

Producing a histogram (Histogram in Excel)

The *Histogram* function in Excel requires a little more effort to master, compared with other chart types. Essentially, a histogram is a graphical display of frequencies (counts) for a continuous quantitative variable (pp. 82, 568), where the data values are grouped into classes. It is possible to select the upper limit for each class into which you want the data to be grouped (these are termed 'bin range values' in Excel). An alternative approach is to let the software select the class intervals (bin range values) for you: Excel selects evenly distributed bins between the minimum and maximum values. However, this is often less effective than selecting your own class intervals.

To use the Excel histogram function, you will need to make sure that the *Analysis ToolPak* is loaded (check under the *Data* tab and, if not loaded, select via the *Office Button* > *Excel Options* > *Add-ins* > *Manage Excel Add-ins* > *Analysis*

ToolPak. The *Histogram* function should then be available in the *Analysis ToolPak* option on the *Data* tab/ribbon.

The following steps outline the procedure used to create the histograms shown in Fig. 74.15 for the table of data below (length, in mm, of 24 leaf petioles from a single plant).

7.2	6.5	7.1	8.5	6.6	7.2
7.0	7.3	8.6	9.1	7.5	8.3
7.1	5.7	7.3	7.6	6.9	7.1
8.3	7.6	5.4	8.6	7.9	8.0

1. **Enter the raw data values**, e.g. as a single column of numbers, or as an array, as above.
2. **Decide on the class intervals to be used.** Base your choice on the number of data points and the maximum and minimum values (for a small data set such as that shown above, you can do this by visual examination, whereas for a large data set, use the Excel functions *COUNT*, *MAX* and *MIN* (find these under the Σ symbol (*More Functions*) on the *Editing* section on the *Home* tab/ribbon, or use the *Descriptive Statistics* > *Summary statistics* option of the *Data Analysis* component on the *Data* tab/ribbon). A typical histogram would have 4–10 classes, depending on the level of discrimination required. Enter the upper limit for each class (*bin range values*) in ascending order in a separate array of cells, e.g. in a column close to the data values (in the above example, 6, 7, 8 and 9 were chosen – the few data values above the final bin value will be shown on the histogram as a group labelled 'more').
3. **Select the histogram function, then input your data and class interval values.** From the *Data* tab/ribbon, select *Data Analysis* > *Histogram*. A new window will open: input your data into the *Input Range* box (highlight the appropriate cells by clicking on the first data value and dragging to the final data value while holding down the left mouse button). Next, input the *Bin Range* values into the appropriate box (if this is left empty, Excel will select default bin range values). Most of the remaining boxes can be left empty, though you must click the last box to get a *Chart Output*, otherwise the software will give the numerical counts for each group, without drawing a histogram. Click *OK* and entries will be created within a new worksheet, showing the upper limits of each group (in a column labelled *Frequency*), plus a poorly constructed chart based on Excel 2007 default settings, as shown in Fig. 74.15(a) (note that the default output is a bar chart, rather than a histogram, since there are gaps between the groups).

(continued)

Box 74.2 (continued)

Example for a histogram (compare Fig. 74.15(a) with Fig. 74.15(b):

- Chart can be resized to increase height, using the 'sizing handles' at the edges of the chart and border line around graph can be removed using *Format Chart Area > Border Color > No line* (menu available by right-clicking within chart area).
- Title and unnecessary legend box can be removed using the *Delete* option.
- Axis scales can be reformatted using the *Format Axis* menu options (e.g. scales, tick marks, line colour).
- X axis labels (class intervals) can be amended by typing directly into the cells containing the bin range values.
- Axis titles can be changed by typing directly into the axis title box (double-click to access).
- Bar colour can be changed (e.g. to grey, with black outline) using the *Format Data Series > Fill and Border Colour* options.
- Bar chart converted to correct histogram format (no gaps between bars) using the *Format Data Series > Series Options*, setting *Gap Width* to 0%.
- Figure legend can be added below figure in Microsoft Word, following copying and pasting of the Excel histogram into a Word file.

Importing an Excel chart into a Word document

One problem encountered with certain Microsoft Office products is that the standard *Cut > Paste* procedure gives a poor-quality figure, with grainy appearance and fuzzy lines/text; similar problems occur using the *Insert* tab/ribbon in some Word versions. The simplest approach is to follow the step-wise procedure below:

1. Select your Excel chart: right-click outside the chart itself, near to the edge, then choose the *Copy* option from the drop-down menu.
2. Open your Word file, go to the *Home* tab/ribbon and select *Paste special > Microsoft Office Word Document Object* from the *Clipboard* options (left-hand side of ribbon).

3. This should give a graph with the same crisp axis/line/text formatting as the original chart in Excel.

The alternative approach is to use Excel to print the entire graph (chart) as a single sheet, and then add this to the print-out from your word-processed document. However, the disadvantage with this approach is that you cannot produce a professional looking figure legend below your graph.

Spreadsheet commands illustrated here may vary among the various versions of Microsoft Office programs and for different spreadsheet products. Check the precise functions and syntax using the insert function menu option (fx button in some versions of Excel).

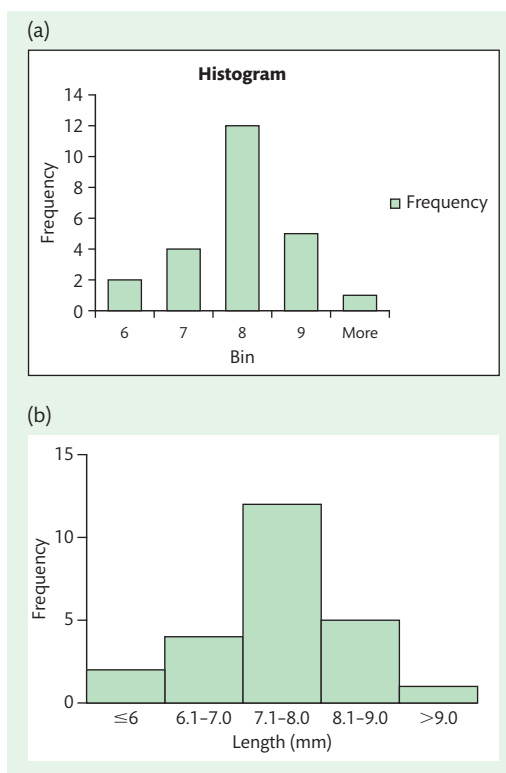


Fig. 74.15 Examples of histogram output from Microsoft Excel using (a) default settings and (b) modified (improved) settings.

Drawing a histogram – each datum is represented by a column with an area proportional to the magnitude of y : in most cases, you should use columns of equal width, so that the height of each column is then directly proportional to y . Shading or stippling may be used to identify individual columns, according to your needs.

The histogram

Whereas a plotted curve assumes a continuous relationship between the variables by interpolating between individual data points, a histogram involves no such assumptions. Histograms are also used to represent frequency distributions (p. 82), where the y axis shows the number of times a particular value of x was obtained (for example, Fig. 74.2). As in a plotted curve, the x axis represents a continuous quantitative variable that can take any value within a given range (for example, plant height), so the scale must be broken down into discrete classes and the scale marks on

the x axis should show either the mid-points (mid-values) of each class, or the boundaries between the classes.

The columns are contiguous (adjacent to each other) in a histogram, in contrast to a bar chart, where the columns are separate because the x axis of a bar chart represents discrete values.

Interpreting graphs

The process of analysing a graph can be split into five phases:

Examining graphs – do not be tempted to look at the data displayed within a graph before you have considered the graph's context, read the legend and decided the scale of each axis.

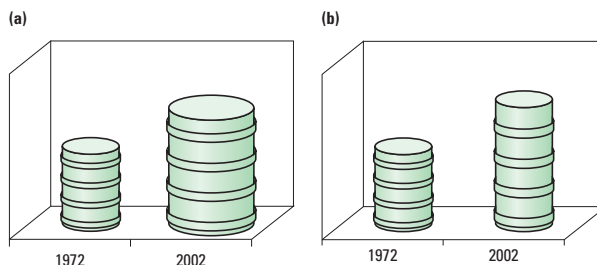
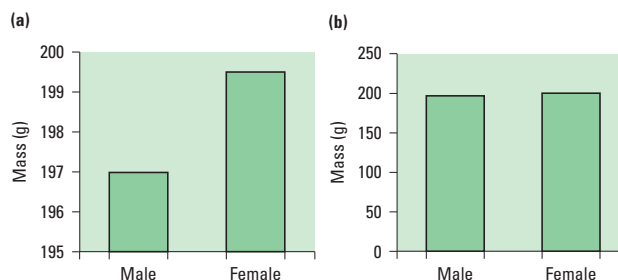
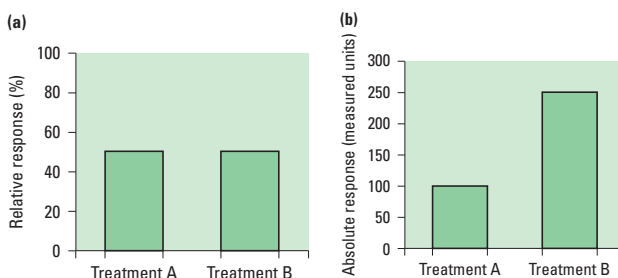
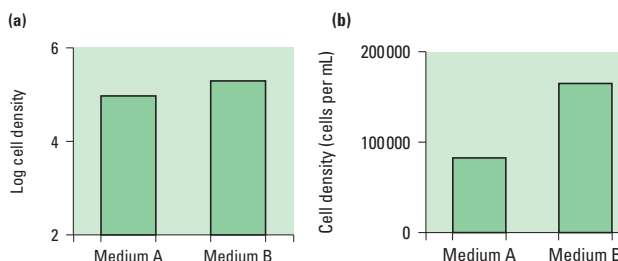
- 1. Consider the context.** Look at the graph in relation to the aims of the study in which it was reported. Why were the observations made? What hypothesis was the experiment set up to test? This information can usually be found in the Introduction or Results section of a report. Also relevant are the general methods used to obtain the results. This might be obvious from the figure title and legend, or from the Materials and Methods section.
- 2. Recognise the graph form and examine the axes.** First, what kind of graph is presented (for example, histogram, plotted curve)? You should be able to recognise the main types summarised on p. 568 and their uses. Next, what do the axes measure? You should check what quantity has been measured in each case and what units are used.
- 3. Look closely at the scale of each axis.** What is the starting point and what is the highest value measured? For the x axis, this will let you know the scope of the treatments or observations (for example, whether they lasted for 5 min or 20 years; whether a concentration span was two-fold or fifty-fold). For each axis, it is especially important to note whether the values start at zero; if not, then the differences between any treatments shown may be magnified by the scale chosen (see Box 74.3).
- 4. Examine the symbols and curves.** Information will be provided in the key or legend to allow you to determine what these refer to. If you have made your own photocopy of the figure, it may be appropriate to note this directly on it. You can now assess what appears to have happened. If, say, two conditions have been observed while a variable is altered, when exactly do they differ from each other; by how much; and for how long?
- 5. Evaluate errors and statistics.** It is important to take account of variability in the data. For example, if mean values are presented, the underlying errors may be large, meaning that any difference between two treatments or observations at a given x -value could simply have arisen by chance. Thinking about the descriptive statistics used (Chapter 76) will allow you to determine whether apparent differences could be significant in both statistical and biological senses.

Understanding graphs within scientific papers – the legend should provide succinct summary of the key information required to interpret the figure without further reference to the main text. Reading this is a useful approach when 'skimming' a paper for relevant information (p. 30).

Sometimes graphs are used to mislead. This may be unwitting, as in an unconscious favouring of a 'pet' hypothesis of the author. Graphs may be used to sell a product in the field of advertising or to favouring of a viewpoint as, perhaps, in politics. Experience in drawing and interpreting graphs will help you spot these flawed presentations, and understanding how graphs can be erroneously presented (Box 74.3) will help you avoid the same pitfalls.

Box 74.3 How graphs can misrepresent and mislead

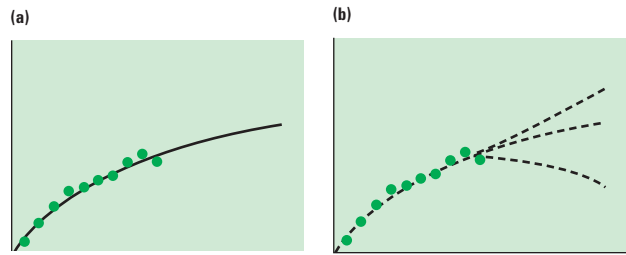
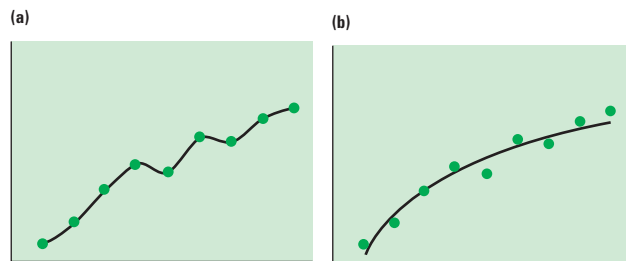
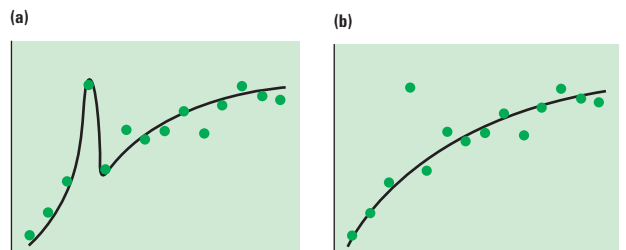
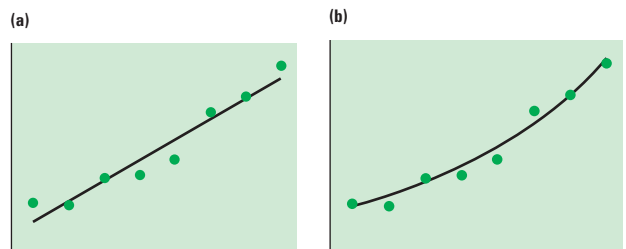
- The 'volume' or 'area' deception** – this is mainly found in histogram or bar chart presentations where the size of a symbol is used to represent the measured variable. For example, the amount of hazardous waste produced in different years might be represented on a chart by different sizes of a chemical drum, with the y axis (height of drum) representing the amount of waste. However, if the symbol retains its *shape* for all heights as in Fig. 74.16(a), its apparent *volume* will increase as a cubic function of the height, rather than in direct proportion. To the casual observer, a two-fold increase may look like an eight-fold one, and so on. Strictly, the *height* of the symbol should be the measure used to represent the variable, with no change in symbol width, as in Fig. 74.16(b).
- The effects of a non-zero axis** – this acts to emphasise the differences between measures by reducing the range of values covered by the axis. For example, in Fig. 74.17(a), it looks as if there are large differences in mass between males and females; however, if the scale is adjusted to run from zero, then it can be seen that the differences are not large as a proportion of the overall mass. Always scrutinise the scale values carefully when interpreting any graph.
- Use of a relative rather than absolute scale** – this is similar to the above, in that data compared using relative scales (e.g. percentage or ratio) can give the wrong impression if the denominator is not the same in all cases. In Fig. 74.18(a), two treatments are shown as equal in *relative* effect, both resulting in 50 relative response compared (say) to the respective controls. However, if treatment A is 50% of a control value of 200 and treatment B is 50% of a control value of 500, then the actual difference in *absolute* response would have been masked, as shown by Fig. 74.18(b).
- The effects of a non-linear scale** – when interpreting graphs with non-linear (e.g. logarithmic) scales, you may interpret any changes on an imagined linear scale. For example, the pH scale is logarithmic, and linear changes on this scale mean less in terms of absolute H^+ concentration at high (alkaline) pH than they do at low (acidic) pH. In Fig. 74.19(a), the cell density in two media is compared on a logarithmic scale, while in Fig. 74.19(b), the same data are graphed on a linear scale. Note, also, that the log y axis scale in Fig. 74.19(a) cannot be shown to zero, because there is no logarithm for 0.

**Fig. 74.16** Increase in pesticide use over a 30-year period.**Fig. 74.17** Average mass of males and females in test group.**Fig. 74.18** Responses to treatments A and B.**Fig. 74.19** Effect of different media on cell density.

(continued)

Box 74.3 (continued)

5. **Unwarranted extrapolation** – a graph may be extrapolated to indicate what would happen if a trend continued, as in Fig. 74.20(a). However, this can be done only under certain assumptions (e.g. that certain factors will remain constant or that relationships will hold under new conditions). There may be no guarantee that this will actually be the case. Figure 74.20(b) illustrates other possible outcomes if the experiment were to be repeated with higher values for the x axis.
6. **Failure to account for data point error** – this misrepresentation involves curves that are overly complex in relation to the scatter in the underlying data. When interpreting graphs with complex curves, consider the errors involved in the data values. It is probably unlikely that the curve would pass through all the data points unless the errors were very small. Figure 74.21(a) illustrates a curve that appears to assume zero error and is thus overly complex, while Fig. 74.21(b) shows a curve that takes possible errors of the points into account.
7. **Failure to reject outlying points** – this is a special case of the previous example. There may be many reasons for outlying data, from genuine mistakes to statistical ‘freaks’. If a curve is drawn through such points on a graph, it indicates that the point carries equal weight with the other points when, in fact, it should probably be ignored. To assess this, consider the accuracy of the measurement, the number and position of adjacent points, and any special factors that might be involved on a one-off basis. Figure 74.22(a) shows a curve where an outlier (arrowed) has perhaps been given undue weight when showing the presumed relationship. If there is good reason to think that the point should be ignored, then the curve shown in Fig. 74.22(b) would probably be more valid.
8. **Inappropriate fitted line** – here, the mathematical function chosen to represent a trend in the data might be inappropriate. A straight line might be fitted to the data, when a curve would be more correct, or vice versa. These cases can be difficult to assess. You need to consider the theoretical validity of the model used to generate the curve (this is not always stated clearly). For example, if a straight line is fitted to the points, the implicit underlying model states that one factor varies in direct relation to another, when the true situation may be more complex. In Fig. 74.23(a), the relationship has been shown as a linear relationship, whereas an exponential relationship, as shown in Fig. 74.23(b), could be more correct.

**Fig. 74.20** Extrapolation of data under different assumptions.**Fig. 74.21** Fitted curves under different assumptions of data error.**Fig. 74.22** Curves with and without outlier taken into account.**Fig. 74.23** Different mathematical model used to represent trends in data.

Sources for further study

Briscoe, M.H. (2013) *Preparing Scientific Illustrations: A Guide to Better Posters, Presentations and Publications*, 2nd edn. Springer-Verlag, New York.

Carter, M. *et al.* *Graphing Resources*. Available: <https://labwrite.ncsu.edu/res/res-homepage.htm>
Last accessed 18/05/21.

[A collection of useful resources including a tutorial on using *Excel* to produce graphs.]

Institute of Biology (2000) *Biological Nomenclature: Recommendations on Terms, Units and Symbols*.
Institute of Biology, London.
[Includes a section on presentation of data.]

IntoBiology. *Get your writing right – symbols and units*. Available: <https://labwrite.ncsu.edu/res/res-homepage.htm>
Last accessed 18/05/21

Robbins, N.B. (2005) *Creating More Effective Graphs*. Wiley, New York.

Yuk, M. and Diamond, S. (2014) *Data Visualization for Dummies*. Wiley, New York.

STUDY EXERCISES

74.1 Select appropriate graphical presentations. Choose an appropriate graphical form for each of the following examples:

- Interaction between pH and cation concentration on enzyme activity.
- Proportion of students with different eye colours in an undergraduate class.
- Relationship between pulse rate and age in humans.
- Number of bacteria per field of view.
- Effect of copper concentration on the activity of an enzyme.

74.2 Create a pie chart. Display the following information in the form of a pie chart. Do *not* use a spreadsheet for this exercise.

Number of bacterial strains isolated from a sample.

Bacterium	Number of strains isolated
<i>Escherichia coli</i>	37
<i>Aeromonas hydrophila</i>	8
<i>Klebsiella spp.</i>	13
<i>Salmonella enterica</i>	2
<i>Enterococcus spp.</i>	25

74.3 Create a frequency distribution histogram. The following table gives data for the haemoglobin levels of 100 people. Plot a histogram showing the frequency distribution of the data. Write a brief description of the important features of the distribution.

Haemoglobin content (g L^{-1}) in blood

11.1	14.2	13.5	9.8	12.0	13.9	14.1	14.6	11.0	12.3
13.4	12.9	12.9	10.0	13.1	11.8	12.6	10.7	8.1	11.2
13.8	12.4	12.9	11.3	12.7	12.4	14.6	15.1	11.2	9.7
11.3	14.7	10.8	13.3	11.9	11.4	12.5	13.0	11.6	13.1
9.3	13.5	14.6	11.2	11.7	10.9	12.4	12.0	12.1	12.6
10.9	12.1	13.4	9.5	12.5	11.6	12.2	8.8	10.7	11.1
10.2	11.7	10.4	14.0	14.9	11.5	12.0	13.2	12.1	13.3
12.4	9.4	13.2	12.5	10.8	11.7	12.7	14.1	10.4	10.5
13.3	10.6	10.5	13.7	11.8	14.1	10.3	13.6	10.4	13.9
11.7	12.8	10.4	11.9	11.4	10.6	12.7	11.4	12.9	12.1

74.4 Find examples of misleading graphs. Create a portfolio of examples of misleading graphs taken from newspapers. For each graph, state what aspect is misleading (see Box 74.3) and, where possible, attempt to show the data correctly in a new graph.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

75 Solving numerical problems

Biology often requires a numerical or statistical approach. Not only is mathematical modelling an important aid to understanding, but computations are also often needed to turn raw data into meaningful information or to compare them with other data sets. Moreover, calculations are part of laboratory routine, perhaps required for making up solutions of known concentration (see p. 156 and text below) or for the calibration of a microscope (see p. 175). In research, ‘trial’ calculations can reveal what input data are required and where errors in their measurement might be amplified in the final result (see pp. 189–90).

KEY POINT If you find numerical work difficult, practice at problem-solving is especially important.

Examples The following boxes give advice on dealing with numerical procedures:

- Box 21.1: preparing solutions (p. 133)
- Box 23.1: molar concentrations (p. 153)
- Box 34.1: cell-counting chambers (p. 245)
- Box 34.2: plate (colony) counts (p. 247)
- Box 43.1: calibration curves (p. 314)
- Box 54.1: radioactivity (p. 405)

Practising numerical problem-solving:

- **demystifies the procedures involved**, which are normally just the elementary mathematical operations of addition, subtraction, multiplication and division (Table 75.1)
- **allows you to gain confidence** so that you do not become confused when confronted with an unfamiliar or apparently complex form of problem
- **helps you recognise the various forms a problem can take** as, for instance, in crossing experiments in classical genetics (Box 65.1).

Table 75.1 Sets of numbers and operations

Sets of numbers

Whole numbers:	0, 1, 2, 3, ...
Natural numbers:	1, 2, 3, ...
Integers:	... -3, -2, -1, 0, 1, 2, 3, ...
Real numbers:	integers and anything between (e.g. -5, 4.376, 3/16, π , $\sqrt{5}$)
Prime numbers:	subset of natural numbers divisible by 1 and themselves only (i.e. 2, 3, 5, 7, 11, 13, ...)
Rational numbers:	p/q where p (integer) and q (natural) have no common factor (e.g. 3/4)
Fractions:	p/q where p is an integer and q is natural (e.g. -6/8)
Irrational numbers:	real numbers with no exact value (e.g. π)
Infinity:	(symbol ∞) is larger than any number (technically not a number as it does not obey the laws of algebra)

Operations and symbols

Basic operators:	+, -, \times and \div will not need explanation; however, / may substitute for \div , * may substitute for \times or this operator may be omitted
Powers:	a^n , i.e. ‘ a to the power n ’, means a multiplied by itself n times (e.g. $a^2 = a \times a =$ ‘ a squared’, $a^3 = a \times a \times a =$ ‘ a cubed’). n is said to be the index or exponent. Note $a^0 = 1$ and $a^1 = a$
Logarithms:	the common logarithm (log) of any number x is the power to which 10 would have to be raised to give x (i.e. the log of 100 is 2; $10^2 = 100$); the antilog of x is 10^x . Note that there is no log for 0, so take this into account when drawing log axes by breaking the axis. Natural or Napierian logarithms (ln) use the base e (= 2.71828 ...) instead of 10
Reciprocals:	the reciprocal of a real number a is $1/a$ ($a \neq 0$)
Relational operators:	$a > b$ means ‘ a is greater (more positive) than b ’, $a < b$ means less than, $a \leq b$ means less-than-or-equal-to and $a \geq b$ means greater-than-or-equal-to
Proportionality:	$a \propto b$ means ‘ a is proportional to b ’ (i.e. $a = kb$, where k is a constant). If $a \propto 1/b$, a is inversely proportional to b ($a = k/b$)
Sums:	$\sum x_i$ is shorthand for the sum of all x values from $i = 0$ to $i = n$ (more correctly the range of the sum is specified under the symbol)
Moduli:	$ x $ signifies modulus of x , i.e. its absolute value (e.g. $ 4 = -4 = 4$)
Factorials:	$x!$ signifies factorial x , the product of all integers from 1 to x (e.g. $3! = 6$). Note $0! = 1! = 1$

Tracing errors in mathematical problems

– this is always easier when all the stages in a calculation are laid out clearly.

Using a spreadsheet for numerical problems

– this may be very useful in repetitive work or for ‘what if?’ case studies (see Chapter 72). When using a spreadsheet, much of the guidance contained in this chapter remains relevant.

Table 75.2 Simple algebra – rules for manipulating

If $a = b + c$, then $b = a - c$ and $c = a - b$
If $a = b \times c$, then $b = a \div c$ and $c = a \div b$
$a = b^c$, then $b = a^{1/c}$ and $c = \log a \div \log b$
$a^{1/n} = \sqrt[n]{a}$
$a^{-n} = 1 \div a^n$
$a^b \times a^c = a^{(b+c)}$ and $a^b \div a^c = a^{(b-c)}$
$(a^b)^c = a^{(a \times c)}$
$a \times b = \text{antilog}(\log a + \log b)$

Presenting calculations in assessed work

– always show the steps in your calculations; most markers will only penalise a mistake once, and part marks will be given if the remaining operations are performed correctly. This can only be done if those operations are visible.

Tackling a numerical problem

The step-by-step approach outlined below may not be the fastest method of arriving at an answer, but most mistakes occur where steps are missing, combined or not made obvious, so a logical approach is often better.

Have the right tools ready

Scientific calculators or spreadsheets (Chapter 72) greatly simplify the numerical part of problem-solving. However, their seeming infallibility may lead you to accept an absurd result that could have arisen because of faulty key-pressing, incorrect application of functions or faulty logic. For calculators, make sure you know how to use all the features, especially how the memory works; how to introduce a constant multiplier or divider; and how to obtain an exponent (note that the ‘exp’ button on most calculators gives you 10^x , not 1^x or y^x ; so 1×10^6 would be entered as $\boxed{1} \boxed{\text{exp}} \boxed{6}$, not $\boxed{10} \boxed{\text{exp}} \boxed{6}$).

Approach the problem thoughtfully

If the individual steps have been laid out on a worksheet, the ‘tactics’ will already have been decided. It is more difficult when you have to adopt a strategy on your own, especially if the problem is presented as a story and it is not obvious which equations or rules need to be applied.

- **Read the problem carefully** as the text may give clues as to how it should be tackled. Be certain of what is required as an answer before starting.
- **Analyse what kind of problem it is**, which effectively means deciding which equation(s) or approach will be applicable. If this is not obvious, consider the dimensions/units of the information available and think how they could be fitted to a relevant formula. In examinations, a favourite ploy of examiners is to present a problem such that the familiar form of an equation must be rearranged (see Table 75.2 and Box 75.1). Another is to make you use two or more equations in series (see Box 75.2). If you are unsure whether a recalled formula is correct, a dimensional analysis can help: write in all the units for the variables and make sure that they cancel out to give the expected answer.
- **Check that you have, or can derive, all of the information required** to use your chosen equation(s). It is unusual but not unknown for examiners to supply redundant information. So, if you decide not to use some of the information given, know why you do not require it.
- **Decide in what format and units the answer should be presented.** This is sometimes suggested to you. If the problem requires many changes in the prefixes to units, it is a good idea to convert all data to base SI units (multiplied by a power of 10) at the outset.
- **If a problem appears complex, break it down into component parts.**

Present your answer clearly

The way you present your answer obviously needs to fit the individual problem. The example shown in Box 75.2 has been chosen to illustrate several important points, but this format would not fit all situations. Guidelines for presenting an answer include:

- **Make your assumptions explicit.** Most mathematical models of biological phenomena require that certain criteria are met before

Box 75.1 How to use algebraic rules

This is best explained by an example showing the application of the rules shown in Table 75.2:

Problem: $a = (b - c) \div (d + e^n)$, find e

1. Multiply both sides by $(d + e^n)$; formula becomes:
 $a(d + e^n) = (b - c)$

2. Divide both sides by a ; formula becomes:

$$d + e^n = \frac{b - c}{a}$$

3. Subtract d from both sides; formula becomes:

$$e^n = \frac{b - c}{a} - d$$

4. Raise each side to the power $1/n$; formula becomes:

$$e = \left(\frac{b - c}{a} - d \right)^{1/n}$$

Providing units – never write any answer without its unit(s) unless it is truly dimensionless.

they can be legitimately applied (for example, ‘assuming the tissue is homogeneous . . .’), while some approaches involve approximations that should be clearly stated (for example, ‘to estimate the mouse’s skin area, its body was approximated to a cylinder with radius x and height y . . .’).

- **Explain your strategy for answering**, perhaps giving the applicable formula or definitions that suit the approach to be taken. Give details of what the symbols mean (and their units) at this point.
- **Rearrange the formula to the required form** with the desired unknown on the left-hand side (see Table 75.2).
- **Substitute the relevant values into the right-hand side of the formula**, using the units and prefixes as given (it may be convenient to convert values to SI beforehand). Convert prefixes to appropriate powers of 10 as soon as possible.
- **Convert to the desired units step by step, i.e. taking each variable in turn.**
- **When you have the answer in the desired units, rewrite the left-hand side and underline the answer for emphasis.** Make sure that the result is presented to an appropriate number of significant figures (see below).

Check your answer

Having written out your answer, you should check it methodically, answering the following questions:

- **Is the answer realistic?** You should be alerted to an error if a number is absurdly large or small. In repeated calculations, a result standing out from others in the same series should be double-checked.
- **Do the units make sense and match up with the answer required?** Do not, for example, present a volume in units of m^2 .
- **Do you get the same answer if you recalculate in a different way?** If you have time, recalculate the answer using a different ‘route’, entering the numbers into your calculator in a different form and/or carrying out the operations in a different order.

Rounding off to a specific number of significant figures – do not round off numbers until you arrive at the final answer or you will introduce errors into the calculation.

Rounding numerical data: decimal places and significant figures

In many instances, the answer you produce as a result of a calculation will include more figures than is justified by the accuracy and precision of the original data. Sometimes you will be asked to produce an answer to a specified number of decimal places or significant figures and other times you will be expected to decide for yourself what would be appropriate.

Box 75.2 How to answer a typical biological problem

This can be illustrated by considering a specific example:

1. Problem

Estimate the total length and surface area of the fibrous roots on a maize seedling from measurements of their total fresh weight and mean diameter. Give your answers in m and cm² respectively to four significant figures.

2. Measurements

Fresh weight^a = 5.00 g, mean diameter^b = 0.5 mm.

3. Answer

Assumptions: (1) the roots are cylinders with constant radius^c and the 'ends' have negligible area; (2) the root system has a density of 1000 kg m⁻³ (i.e. that of water^d).

Strategy: from assumption (1), the applicable equations are those concerned with the volume and surface area of a cylinder (Table 75.3), namely:

$$V = \pi r^2 h \quad [75.1]$$

$$A = 2\pi rh \text{ (ignoring eds)} \quad [75.2]$$

where V is volume (m³), A is surface area (m²), $\pi \approx 3.14159$, h is height (m) and r is radius (m). The total length of the root system is given by h and its surface area by A . We can find h by rearranging eqn [75.1] and then substitute its value in eqn [75.2] to get A .

To calculate total root length: rearranging eqn [75.1], we have $h = V/\pi r^2$. From measurements^e, $r = 0.25 \text{ mm} = 0.25 \times 10^{-3} \text{ m}$

From density = weight/volume,

$$\begin{aligned} V &= \text{fresh weight/density} \\ &= 5 \text{ g}/1000 \text{ kg m}^{-3} \\ &= 0.005 \text{ kg}/1000 \text{ kg m}^{-3} \\ &= 5 \times 10^{-6} \text{ m}^3 \end{aligned}$$

Total root length,

$$h = V/\pi r^2$$

$$5 \times 10^{-6} \text{ m}^3 / 3.14159 \times (0.25 \times 10^{-3} \text{ m})^2$$

$$\therefore \text{Total root length} = 25.46 \text{ m}$$

To calculate surface area of roots: substituting value for h obtained above into eqn [75.2], we have:

Root surface area

$$\begin{aligned} &= 2 \times 3.14159 \times 0.25 \times 10^{-3} \text{ m} \times 25.46 \text{ m} \\ &= 0.04 \text{ m}^2 \end{aligned}$$

$$= 0.04 \times 10^4 \text{ cm}^2$$

(there being $100 \times 100 = 10^4 \text{ cm}^2 \text{ per m}^2$)

$$\therefore \text{Root surface area} = 400.0 \text{ cm}^2$$

4. Notes

- The fresh weight of roots would normally be obtained by washing the roots free of soil, blotting them dry and weighing.
- In a real answer you might show the replicate measurements giving rise to the mean diameter.
- In reality, the roots will differ considerably in diameter and each root will not have a constant diameter throughout its length.
- This will not be wildly inaccurate as about 95% of the fresh weight will be water, but the volume could also be estimated from water displacement measurements.
- Note conversion of measurements into base SI units at this stage and on line 3 of the root volume calculation. Forgetting to halve diameter measurements where radii are required is a common error.

KEY POINT Do not simply accept the numerical answer from a calculator or spreadsheet, without considering whether you need to modify this to give an appropriate number of significant figures or decimal places.

Rounding to n decimal places

This is relatively easy to do.

- Look at the number to the right of the n th decimal place.
- If this is less than five, simply 'cut off' all numbers to the right of the n th decimal place to produce the answer (i.e. round down).

Examples

The number 4.123 correct to two decimal places is 4.12

The number 4.126 correct to two decimal places is 4.13

The number 4.1251 correct to two decimal places is 4.13

The number 4.1250 correct to two decimal places is 4.12

The number 4.1350 correct to two decimal places is 4.14

The number 99.99 correct to one decimal place is 100.0.

Examples

The number of significant figures in 194 is 3

The number of significant figures in 2305 is 4

The number of significant figures in 0.003482 is 4

The number of significant figures in 210×10^8 is 3 (21×10^9 would be 2).

Examples

The number of significant figures in 3051.93 is 6

To five significant figures, this number is 3051.9

To four significant figures, this number is 3052

To three significant figures, this number is 3050

To two significant figures, this number is 3100

To one significant figure, this number is 3000

3051.93 to the nearest 10 is 3050

3051.93 to the nearest 100 is 3100

Note that in this last case you must include the zeros before the decimal point to indicate the scale of the number (even if the decimal point is not shown). For a number less than 1, the same would apply to the zeros before the decimal point. For example, 0.00305193 to three significant figures is 0.00305. Alternatively, use scientific notation (in this case, 3.05×10^{-3}).

3. If the number is greater than five, 'cut off' all numbers to the right of the n th decimal place and add one to the n th decimal place to produce the answer (i.e. round up).
4. If the number is 5, then look at further numbers to the right to determine whether to round up or not.
5. If the number is exactly 5 and there are no further numbers to the right, then round to the nearest even number. *Note:* When considering a large number of calculations, this procedure will not affect the overall mean value. Some rounding systems do the opposite to this (i.e. round to the nearest odd number), while others always round up where the number is exactly 5 (which *will* affect the mean). Take advice from your tutor and stick to one system throughout a series of calculations.

Whenever you see any number quoted, you should assume that the last digit has been rounded. For example, in the number 22.4, the '.4' is assumed to be rounded and the calculated value may have been between 22.35 and 22.45.

Quoting to n significant figures

The number of significant figures indicates the degree of approximation in the number. For most cases, it is given by counting all the figures except zeros that occur at the beginning or end of the number. Zeros *within* the number are always counted as significant. The number of significant figures in a number like 200 is ambiguous and could be one, two or three; if you wish to specify clearly, then quote as for example, 2×10^2 (one significant figure), 2.0×10^2 (two significant figures), etc. to avoid spurious accuracy (p. 189). When quoting a number to a specified number of significant figures, use the same rules as for rounding to a specified number of decimal places, but do not forget to keep zeros before or after the decimal point. The same principle is used if you are asked to quote a number to the 'nearest 10', 'nearest 100', etc.


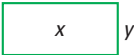

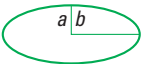
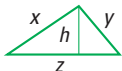
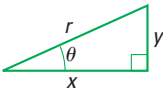
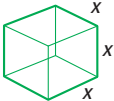
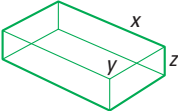
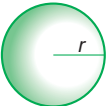
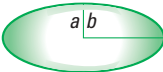
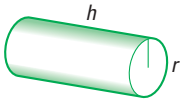
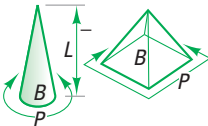
When deciding for yourself how many significant figures to use, adopt the following rules of thumb:

- **Always round *after* you have done a calculation.** Use *all* significant figures available in the measured data during a calculation.
- **If adding or subtracting with measured data, then quote the answer to the number of decimal places in the data value with the least number of decimal places** (for example, $32.1 - 45.67 + 35.6201 = 22.1$, because 32.1 has one decimal place).
- **If multiplying or dividing with measured data, keep as many significant figures as are in the number with the least number of significant places** (for example, $34901 \div 3445 \times 1.3410344 = 13.59$, because 3445 has four significant figures).
- **For the purposes of significant figures, assume 'constants' have an infinite number of significant figures** (for example, number of mm in a metre).

Applying the rules of mathematics

Errors in calculations sometimes appear because of faults in mathematics rather than computational errors. For reference purposes, Tables 75.1–75.3 give some basic mathematical principles that may be useful. Eason *et al.* (1992) or Stephenson (2010) should be consulted for more advanced/specific needs.

Table 78.3 Geometry and trigonometry – analysing shapes

Shape/object	Diagram	Perimeter	Area
<i>Two-dimensional shapes</i>			
Square		$4x$	x^2
Rectangle		$2(x + y)$	xy
Circle		$2\pi r$	πr^2
Ellipse		$\pi[1.5(a + b) - \sqrt{a*b}]$ (approx.)	πab
Triangle (general)		$x + y + z$	$0.5zh$
(right-angled)		$x + y + r$ $\sin \theta = y/r, \cos \theta = x/r,$ $\tan \theta = y/x, r^2 = x^2 + y^2$	$0.5xy$
Shape/object	Diagram	Surface area	Volume
<i>Three-dimensional shapes</i>			
Cube		$6x^2$	x^3
Cuboid		$2xy + 2xz + 2yz$	xyz
Sphere		$4\pi r^2$	$4\pi r^3/3$
Ellipsoid		no simple formula	πrab
Cylinder		$2\pi rh + 2\pi r^2$	$\pi r^2 h$
Cone and pyramid		$0.5PL + B$	$BL/3$

Key: x, y, z = sides a, b = half minimum and maximum axes; r = radius or hypotenuse; h = height; B = base area; L = perpendicular height; P = perimeter of base.

Examples

1/8 as a percentage is

$$1 \div 8 \times 100 = 100 \div 8 = 12.5\%$$

0.602 as a percentage is $0.602 \times 100 = 60.2\%$

Examples

190% as a decimal fraction is

$$190 \div 100 = 1.9$$

5/2 as a percentage is $5 \div 2 \times 100 = 250\%$

Example A population falls from 4 million to 3.85 million. What is the percentage change? The decrease in numbers is $4 - 3.85 = -0.15$ million.

The fractional decrease is $-0.15 \div 4 = -0.0375$ and we multiply by 100 to get the percentage change = minus 3.75%.

Example $2^3 = 2 \times 2 \times 2 = 8$.

Example Avogadro's number, $\approx 602\,352\,000\,000\,000\,000\,000$, is more conveniently expressed as 6.02352×10^{23} .

Examples The logarithm to the base 10 (\log_{10}) of 1000 is 3, since $10^3 = 1000$. The logarithm to the base e (\log_e or \ln) of 1000 is 6.907755 (to six decimal places).

Percentages

A percentage is just a fraction (Table 75.1) expressed in terms of hundredths, indicated by putting the percentage sign (%) after the number of hundredths. So 35% simply means 35 hundredths. To convert a fraction to a percentage, just multiply the fraction by 100. When the fraction is in decimal form, multiplying by 100 to obtain a percentage is easily achieved just by moving the decimal point two places to the right.

To convert a percentage to a fraction, just remember that, since a percentage is a fraction multiplied by 100, the fraction is the percentage divided by 100. For example: $42\% = 42/100 = 0.42$. In this example, since we are dealing with a decimal fraction, the division by 100 is just a matter of moving the decimal point two places to the left (42% could be written as 42.0%). Percentages greater than 100% represent fractions greater than 1. Percentages less than 1 may cause confusion. For example, 0.5% means half of one per cent (0.005) and must not be confused with 50% (which is the decimal fraction 0.5).

To find a percentage of a given number, just express the percentage as a decimal fraction and multiply the given number. For example: 35% of 500 is given by $0.35 \times 500 = 175$. To find the percentage change in a quantity, work out the difference (=value 'after'–value 'before'), and divide this difference by the original value to give the fractional change, then multiply by 100.

Exponents

Exponential notation is an alternative way of expressing numbers in the form a^n (' a to the power n '), where a is multiplied by itself n times. The number a is called the base and the number n the exponent (or power or index). The exponent need not be a whole number, and it can be negative if the number being expressed is less than 1. See Table 75.2 for other mathematical relationships involving exponents.

Scientific notation

In scientific notation, also known as 'standard form', the base is 10 and the exponent a whole number. To express numbers that are not whole powers of 10, the form $c \times 10^n$ is used, where the coefficient c is normally between 1 and 10. Scientific notation is valuable when you are using very large numbers and wish to avoid suggesting spurious accuracy. Thus if you write 123 000, this may suggest that you know the number to ± 0.5 , whereas 1.23×10^5 might give a truer indication of measurement accuracy (i.e. implied to be ± 500 in this case). Engineering notation is similar, but treats numbers as powers of 10 in groups of 3, i. e. $c \times 10^0, 10^3, 10^6, 10^9$, etc. This corresponds to the SI system of prefixes (p. 192).

A useful property of powers when expressed to the same base is that when multiplying two numbers together, you simply add the powers, while if dividing, you subtract the powers. Thus, suppose you counted 8 bacteria in a known volume of a 10^{-7} dilution there would be 8×10^7 in the same volume of undiluted solution; if you now dilute this 500-fold (5×10^2), then the number present in the same volume would be $8/5 \times 10^{(7-2)} = 1.6 \times 10^5 = 160\,000$.

Logarithms

When a number is expressed as a logarithm, this refers to the power n that the base number a must be raised to give that number. Any base could be

Examples (use to check the correct use of your own calculator)

102 963 as a $\log_{10} = 5.012681$ (to six decimal places)

$10^{5.012681} = 102\,962.96$

(Note loss of accuracy due to loss of decimal places.)

102 963 as a natural logarithm

(\ln) = 11.542125 (to six decimal places), thus

$2.718282^{11.542125} = 102\,963$.

used, but the two most common are 10, when the power is referred to as \log_{10} or simply \log , and the constant e (2.718282), used for mathematical convenience in certain situations, when the power is referred to as \log_e or \ln (natural logarithm). Note that (a) logs need not be whole numbers; (b) there is no \log value for the number zero; and (c) that $\log_{10} = 0$ for the number 1.

To obtain logs, you will need to use the \log key on your calculator, or special log tables (now largely redundant). To convert back (antilog), use

- the 10^x key, with $x = \log$ value; =
- the $\frac{1}{\square}$ then the \log key; or
- the y^x key, with $y = 10$ and $x = \log$ value.

If you have used log tables, you will find complementary antilogarithm tables to do this.

There are many uses of logarithms in biology, including pH ($= -\log[H^+]$), where $[H^+]$ is expressed in mol L^{-1} (see p. 161); the exponential growth of micro-organisms, where if $\log(\text{cell number})$ is plotted against time, a straight-line relationship is obtained (see pp. 242–3); and allometric studies of growth and development, where if data are plotted on log axes, a series of straight-line relationships may be found (see p. 242).

Using linear functions and straight-line relationships

One of the most straightforward and widely used relationships between two variables x and y is that represented by a straight-line graph, where the corresponding mathematical function is known as the equation of a straight line, where:

$$y = a + bx \quad [75.3]$$

In this relationship, a represents the intercept of the line on the y (vertical) axis, i.e. where $x = 0$, and therefore $bx = 0$, while b is equivalent to the slope (gradient) of the line, i.e. the change in y for a change in x of 1. The constants a and b are sometimes given alternative symbols, but the mathematics remains unchanged, for example, in the equivalent expression for the slope of a straight line, $y = mx + c$. Figure 75.1 shows what happens when these two constants are changed, in terms of the resultant straight lines.

The two main applications of the straight-line relationship are:

1. Function fitting. Here, you determine the mathematical form of the function, i.e. you estimate the constants a and b from a data set for x and y , either by drawing a straight line by eye and then working out the slope and y intercept, or by using linear regression (pp. 316, 611) to obtain the most probable values for both constants. When putting a straight line of best fit by eye on a hand-drawn graph, note the following:

- Always use a *transparent* ruler, so you can see data points on either side of the line.
- For a data series where the points do not fit a perfect straight line, try to have an equal number of points on either side of the line, as in (Fig. 75.2(a)), and try to minimise the average distance of these points from the line.

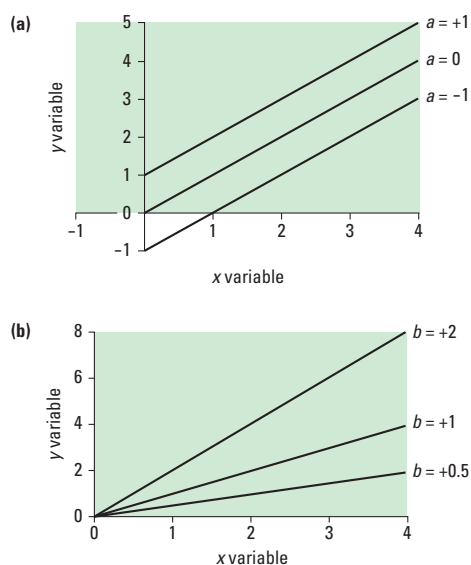


Fig. 75.1 Straight-line relationships ($y = a + bx$), showing the effects of (a) changing the intercept at constant slope, and (b) changing the slope at constant intercept.

Examples Using eqn [75.3], the predicted value for y for a linear function where $a = 2$ and $b = 0.5$, where $x = 8$ is:
 $y = 2 + (0.5 \times 8) = 6$.
 Using eqn [75.4], the predicted value for x for a linear function where $a = 1.5$ and $b = 2.5$, where $y = -8.5$ is:
 $x = (-8.5 - 1.5) \div 2.5 = -4$.
 Using eqn [75.4] the predicted x intercept for a linear function where $a = 0.8$ and $b = 3.2$ is: $x = (0 - 0.8) \div 3.2 = -0.25$.

Example A lab schedule states that 5 g of a compound with a relative molecular mass of 220 are dissolved in 400 mL of solvent. For writing up your Materials and Methods, you wish to express this as mol L^{-1} .

1. If there are 5 g in 400 mL, then there are $5 \div 400$ g in 1 mL.
2. Hence, 1000 mL will contain $5 \div 400 \times 1000$ g = 12.5 g.
3. $12.5 \text{ g} = 12.5 \div 220 \text{ mol} = 0.0568 \text{ mol}$, so $[\text{solution}] = 56.8 \text{ mmol L}^{-1}$ ($= 56.8 \text{ mol m}^{-3}$).

Examples For a geometric dilution series involving ten-fold dilution steps, calculation of concentrations is straightforward, e.g. two serial decimal dilutions ($= 100$ -fold dilution) of a solution of NaCl of 250 mmol L^{-1} will produce a dilute solution of $250 \div 100 = 2.5 \text{ mmol L}^{-1}$. Similarly, for an arithmetic dilution series, divide by the overall dilution to give the final concentration, e.g. a sixteen-fold dilution of a solution of NaCl of 200 mg mL^{-1} will produce a dilute solution of $200 \div 16 = 12.5 \text{ mg mL}^{-1}$.

- Once you have drawn the line of best fit, use this line, rather than your data values, in all subsequent procedures (for example, in a calibration curve, Chapter 43).
- Tangents drawn to a curve give the slope (gradient) at a particular point, for example, in an enzyme reaction progress curve (Fig. 62.3). These are best drawn by bringing your ruler up to the curve at the exact point where you wish to estimate the slope and then trying to make the two angles immediately on either side of this point approximately the same, by eye (Fig. 75.2(b)).
- Once you have drawn the straight line or tangent, choose two points reasonably far apart at either end of your line and then draw construction lines to represent the change in y and the change in x between these two points: make sure that your construction lines are perpendicular to each other. Determine the slope as the change in y divided by the change in x (Fig. 75.2).

2. **Prediction.** Where a and b are known, or have been estimated, you can use eqn [75.3] to predict any value of y for a specified value of x , for example, during exponential growth of a cell culture (pp. 242–3), where \log_{10} cell number (y) increases as a linear function of time (x): note that in this example the dependent variable has been transformed to give a linear relationship. You will need to rearrange eqn [75.3] in cases where a prediction of x is required for a particular value of y (for example, in calibration curves, pp. 313–15, or bioassays, pp. 246–8), as follows:

$$x = (y - a) \div b \quad [75.4]$$

This equation can also be used to determine the intercept on the x (horizontal) axis, i.e. where $y = 0$.

Answering typical problems

Calculations involving proportions or ratios

The ‘unitary method’ is a useful way of approaching calculations involving proportions or ratios, such as those required when making up solutions from stocks (see also Chapter 23) or as a subsidiary part of longer calculations.

1. If given a value for a multiple, work out the corresponding value for a single item or unit.
2. Use this ‘unitary value’ to calculate the required new value.

Calculations involving series

Series (used in for example, dilutions, see also pp. 147–8) can be of three main forms:

1. **Arithmetic**, where the *difference* between two successive numbers in the series is a constant, for example, 2, 4, 6, 8, 10, . . .
2. **Geometric**, where the *ratio* between two successive numbers in the series is a constant, for example, 1, 10, 100, 1000, 10000, . . .
3. **Harmonic**, where the values are reciprocals of successive whole numbers, for example, $1, \frac{1}{2}, \frac{1}{3}, \frac{1}{4}, \dots$

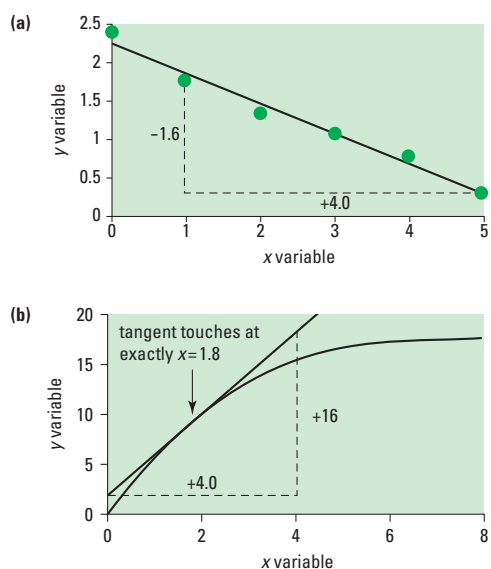


Fig. 75.2 Drawing straight lines. (a) Simple linear relationship, giving a straight line with an intercept of 2.3 and a slope of $-1.6 \div 4.0 = 0.4$. (b) Tangent drawn to a curve at $x = 1.8$, giving a slope of $16 \div 4 = 4$.

Note that the logs of the numbers in a geometric series will form an arithmetic series (for example, 0, 1, 2, 3, 4, . . . in the above case). Thus, if a quantity y varies with a quantity x such that the rate of change in y is proportional to the value of y (i.e. it varies in an exponential manner), a semi-log plot of such data will form a straight line. This form of relationship is relevant for exponentially growing cell cultures (pp. 242–3) and radioactive decay (p. 403).

Statistical calculations

The need for long, complex calculations in statistics has largely been removed because of the widespread use of spreadsheets with statistical functions (Chapter 72) and specialised programs such as *SPSS* and *Minitab*. It is, however, important to understand the principles behind what you are trying to do (see Chapters 76 and 77) and interpret the program's output correctly, either using the 'help' function or a reference manual.

Problems in Mendelian genetics

These cause difficulties for many students. The key is to recognise the different types of problems and to practise so you are familiar with the techniques for solving them. Chapter 65 deals with the different types of crosses you will come across and methods of analysing them, including the use of the χ^2 (chi²) test.

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STUDY EXERCISES

75.1 Rearrange the following formulae.

- (a) If $y = ax + b$, find b
- (b) If $y = ax + b$, find x
- (c) If $x = y^3$, find y
- (d) If $x = 3^y$, find y
- (e) If $x = (1 - y)(z^p + 3)$, find z
- (f) If $x = (y - z)^{1/n}/pq$, find n

75.2 Work with decimal places or significant figures.

Give the following numbers to the accuracy indicated:

- (a) 214.51 to three significant figures
- (b) 107 029 to three significant figures
- (c) 0.0450 to one significant figure
- (d) 99.817 to two decimal places
- (e) 99.897 to two decimal places
- (f) 99.997 to two decimal places
- (g) 6255 to the nearest 10
- (h) 134 903 to the nearest ten thousand

State the following:

- (i) the number of significant figures in 3400
- (ii) the number of significant figures in 3400.3
- (iii) the number of significant figures in 0.00167
- (iv) the number of significant figures in 1.00167
- (v) the number of decimal places in 34.46
- (vi) the number of decimal places in 0.00167

75.3 Carry out calculations involving percentages.

Answer the following questions, giving your answers to two decimal places:

- (a) What is 6/35ths expressed as a percentage?
- (b) What is 0.0755 expressed as a percentage?
- (c) What is 4.35% of 322?
- (d) A rat's weight increased from 55.23 g to 75.02 g. What is the percentage increase in its weight?

75.4 Practise using exponents and logarithms.

- (a) Write out (i) the charge on an electron and (ii) the speed of light *in vacuo* in longhand (i.e. without using powers of 10). See Table 28.4 for values given in scientific notation.
- (b) Compute the following values: 10^{3624} ; $\log(6.37)$; $e^{-2.32}$; $\ln(1123)$; $6^{3.2}$. A calculator should be used, but round the output to give five significant figures.

75.5 Practise using geometric formulae. Select appropriate formulae from Table 75.3, and use them to compare the surface-area-to-volume ratios of the following:

- (a) a spherical unicellular alga of diameter 30 μm ;
- (b) a cylindrical root of radius 0.5 mm (ignoring ends).

75.6 Practise working with linear functions (note also that Chapter 43 includes study exercises based on linear functions and plotting straight lines). Assuming a linear relationship between x and y , calculate the following (give your answers to three significant figures):

- (a) x , where $y = 7.0$, $a = 4.5$ and $b = 0.02$;
- (b) x , where $y = 15.2$, $a = -2.6$ and $b = -4.46$;
- (c) y , where $x = 10.5$, $a = 0.2$ and $b = -0.63$;
- (d) y , where $x = 4.5$, $a = -1.8$ and $b = 4.1$.

Answers to these study exercises are available at go.pearson.com/uk/he/resources

76 Using descriptive statistics

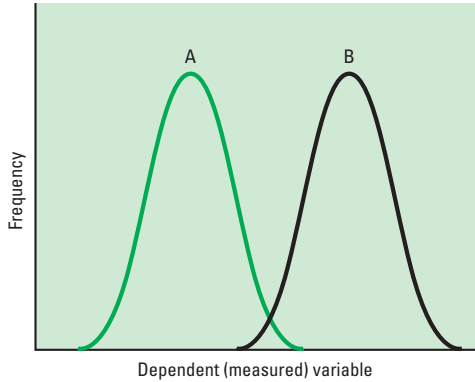


Fig. 76.1 Two distributions with different locations but the same dispersion. The data set labelled B could have been obtained by adding a constant to each datum in the data set labelled A.

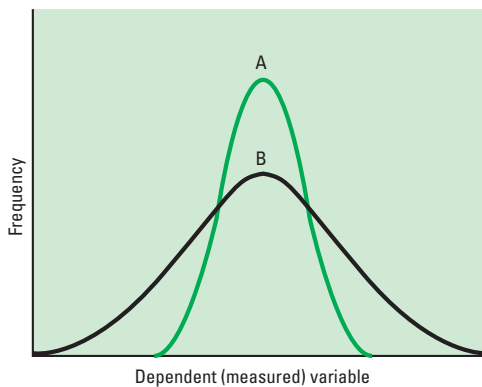


Fig. 76.2 Two distributions with different dispersions but the same location. The data set labelled A covers a relatively narrow range of values of the dependent (measured) variable, while that labelled B covers a wider range.

Example Box 76.1 shows a set of data and the calculated values of the measures of location, dispersion and shape for which methods of calculation are outlined here. Check your understanding by calculating the statistics yourself and confirming that you arrive at the same answers.

Whether obtained from observation or experimentation, most data in biology exhibit variability. You can display this variability as a frequency distribution (for example, Fig. 74.2). Descriptive (or summary) statistics quantify aspects of these frequency distributions. You can use them to:

- **condense a large data set** for presentation in figures or tables
- **provide estimates of parameters** of the frequency distribution of the population being sampled (p. 548).

The three important features of a frequency distribution that you can summarise with descriptive statistics are:

- **the location of the data set**, i.e. its position along a given dimension representing the (measured) variable (Fig. 76.1)
- **the dispersion of the data**, i.e. how spread out the values are (Fig. 76.2)
- **the shape of the frequency distribution**, i.e. whether symmetrical, skewed, U-shaped, etc. (Fig. 76.3).

KEY POINT Descriptive statistics are generally used to provide estimates of population parameters from a representative sample of the population. It is usually assumed that the replicates in an experiment will qualify as this kind of sample.

The appropriate statistics you should use depend on both the type of data (i.e. whether quantitative, ranked or qualitative, pp. 594–5) and the nature of the underlying frequency distribution. You may be able to predict a particular type of frequency distribution based on prior knowledge or theoretical considerations. If not, it can be determined graphically from a representative sample (preferably with $n > 100$) or via statistical tests (Chapter 77). Where data sets do not fit a ‘standard’ distribution (for example, a bimodal shape, p. 597), more complex descriptions (or further analysis) may be required.

The methods you should use to calculate descriptive statistics also depend on whether the data have been grouped into classes. Use the

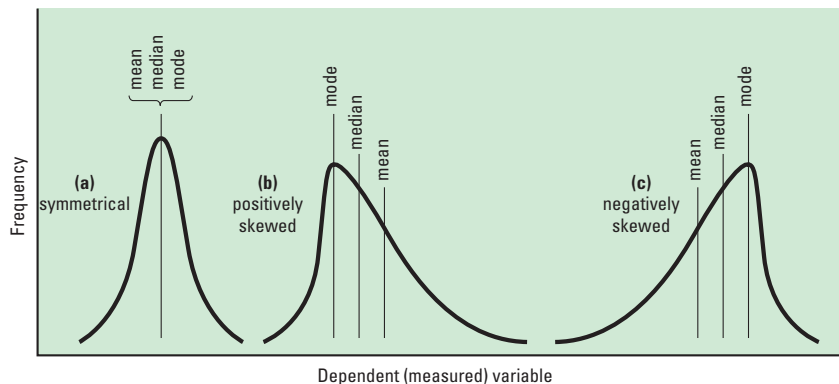


Fig. 76.3 Symmetrical and skewed frequency distributions, showing relative positions of mean, median and mode.

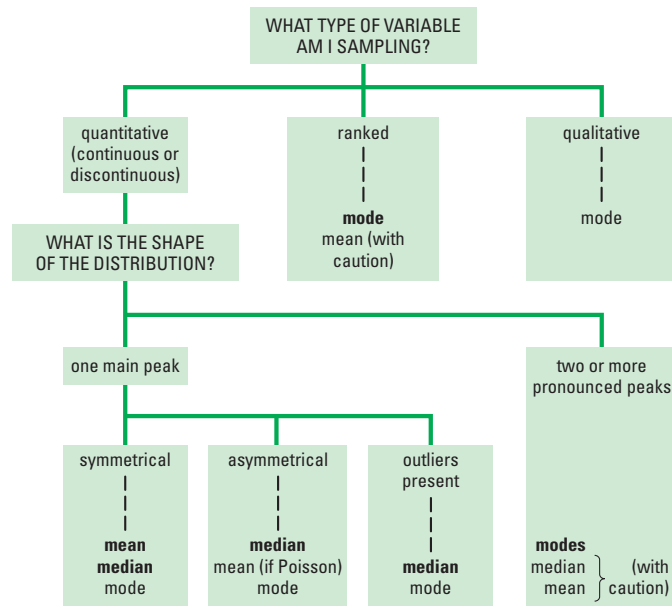


Fig. 76.4 Choosing a statistic for characterising a distribution's location. Statistics written in bold are the preferred option(s).

Using symbols for statistics – Y is used in Chapters 76 and 77 to signify the dependent variable in statistical calculations (following the example of Sokal and Rohlf, 2012, Heath, 1995 and Wardlaw, 2000). Note, however, that some authors use X or x in analogous formulae and many calculators refer to \bar{x} , $\sum x^2$, etc for their statistical functions.

Definition

Rank – the position of a data value when all the data are placed in order of ascending magnitude. If ties occur, an average rank of the tied variates is used. Thus, the rank of the datum 6 in the sequence 1, 3, 5, 6, 8, 8, 10 is 4; the rank of each datum with value 8 is 5.5.

Definition

An outlier – any datum that has a value much smaller or bigger than most of the data.

original data set if at all possible, because grouping loses information and reduces accuracy. Large data sets are best handled using computer software.

Measuring location

Here, the objective is to pinpoint the 'centre' of the frequency distribution, i.e. the value about which most of the data are grouped. The chief measures of location you should consider are the mean, median and mode. Figure 76.4 shows how to choose among these for a given data set, depending on the type of variable being measured (Chapter 27).

Mean

This is the average value of the data (denoted \bar{Y} and also referred to as the arithmetic mean). It is obtained from the sum of all the data values divided by the number of observations (in symbolic representation, $\sum Y/n$). The mean is a good measure of the centre of symmetrical frequency distributions. It uses all of the numerical values of the sample and therefore incorporates all of the information content of the data. However, the value of a mean is greatly affected by the presence of outliers (extreme values). The arithmetic mean is a widely used statistic in biology, but there are situations where you should not use it (see Box 76.2 for examples).

Median

This is the mid-point of the observations when ranked in increasing order. For odd-sized samples, the median is the middle observation; for even-sized samples it is the mean of the middle pair of observations. Where data are grouped into classes, you can only estimate the median. This is most simply done from a graph of the cumulative frequency distribution, but can also be calculated by assuming the data to be evenly spread within the class. The median represents the location of the main body of data better than the mean when the distribution is asymmetric or when there are outliers in the sample.

Box 76.1 How to use descriptive statistics – an example

Value (Y)	Frequency (f)	Cumulative frequency	fY	fY ²
1	0	0	0	0
2	1	1	2	4
3	2	3	6	18
4	3	6	12	48
5	8	14	40	200
6	5	19	30	180
7	2	21	14	98
8	0	21	0	0
Totals	21 = $\sum f (=n)$		104 = $\sum fY$	548 = $\sum fY^2$

In this example, for simplicity and ease of calculation, integer values of Y are used. In many practical exercises, where continuous variables are measured to several significant figures and where the number of data values is small, giving frequencies of 1 for most of the values of Y, it may be simpler to omit the column dealing with frequency and list all the individual values of Y and Y² in the appropriate

columns. To gauge the underlying frequency distribution of such data sets, you would need to group individual data into broader classes (e.g. all values between 1.0 and 1.9, all values between 2.0 and 2.9, etc.) and then draw a histogram (p. 575). Calculation of certain statistics for data sets that have been grouped in this way (e.g. median, quartiles, extremes) can be tricky and a statistical text should be consulted.

Statistic	Value*	How calculated
Mean	4.95	$\sum fY/n$, i.e. 104/21
Median	5	Value of the $(n + 1)/2$ variate, i.e. the value ranked $(21 + 1)/2 = 11$ th (obtained from the cumulative frequency column)
Mode	5	The most common value (Y value with highest frequency)
Upper quartile	6	The upper quartile is between the 16th and 17th values, i.e. the value exceeded by 25% of the data values
Lower quartile	4	The lower quartile is between the 5th and 6th values, i.e. the value exceeded by 75% of the data values
Semi-interquartile range	1.0	Half the difference between the upper and lower quartiles, i.e. $(6 - 4)/2$
Upper extreme	7	Highest Y value in data set
Lower extreme	2	Lowest Y value in data set
Range	5	Difference between upper and lower extremes
Variance (s^2)	1.65	$s^2 = \frac{\sum fY^2 - [(\sum fY)^2/n]}{n - 1}$ $= \frac{548 - (104)^2/21}{20}$
Standard deviation (s)	1.28	$\sqrt{s^2}$
Standard error (SE)	0.280	s/\sqrt{n}
95% confidence limits	4.36 – 5.54	$\bar{Y} \pm t_{0.05}[20] \times SE$, (where $t_{0.05}[20] = 2.09$, Table 76.2)
Coefficient of variation (CoV)	25.9%	$100s/\bar{Y}$

*Rounded to three significant figures except when it is an exact number.

Describing the location of qualitative data – the mode is the only statistic that is suitable for this task. For example, ‘the modal (most frequent) *Drosophila* eye colour was crimson’.

Mode

This is the most common value in the sample. You can find the mode easily from a tabulated frequency distribution as the most frequent value. Where data have been grouped into classes then the term ‘modal class’ is used for the class containing most values. The mode provides a rapidly and easily found estimate of sample location and is unaffected by outliers. However, the mode is affected by chance variation in the shape of a sample’s distribution and it may lie distant from the obvious centre of the distribution.

The mean, median and mode have the same units as the variable being measured. However, whether these statistics of location have the same or similar values for a given frequency distribution depends on the symmetry and shape of the frequency distribution. If it is near-symmetrical with a single peak, all three will be very similar; if it is skewed or has more than one peak, their values will differ to a greater degree (see Fig. 76.3).

Measuring dispersion

Here, your objective is to quantify the spread of the data about the centre of the distribution. Figure 76.5 provides practical advice on how to decide which measure of dispersion to use.

Range

This is the difference between the largest and smallest data values in the sample (the extremes) and has the same units as the measured variable. The range is easy to determine, but is greatly affected by outliers. Its value may also depend on sample size: in general, the larger this is, the greater will be the range. These features make the range a poor measure of dispersion for many practical purposes.

Semi-interquartile range

This is an appropriate measure of dispersion if you are working with a data set where the median is the appropriate statistic to describe location. For this,

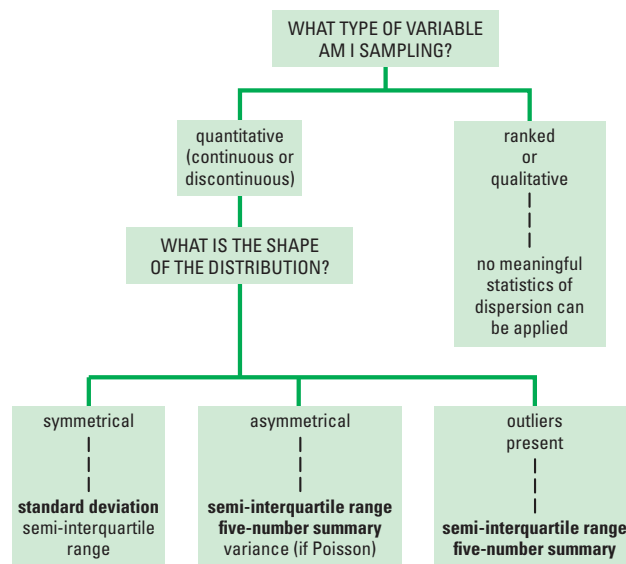


Fig. 76.5 Choosing a statistic for characterising a distribution’s dispersion. Statistics written in bold are the preferred option(s). Note that you should match statistics describing dispersion with those you have used to describe location, i.e. standard deviation with mean, semi-interquartile range with median.

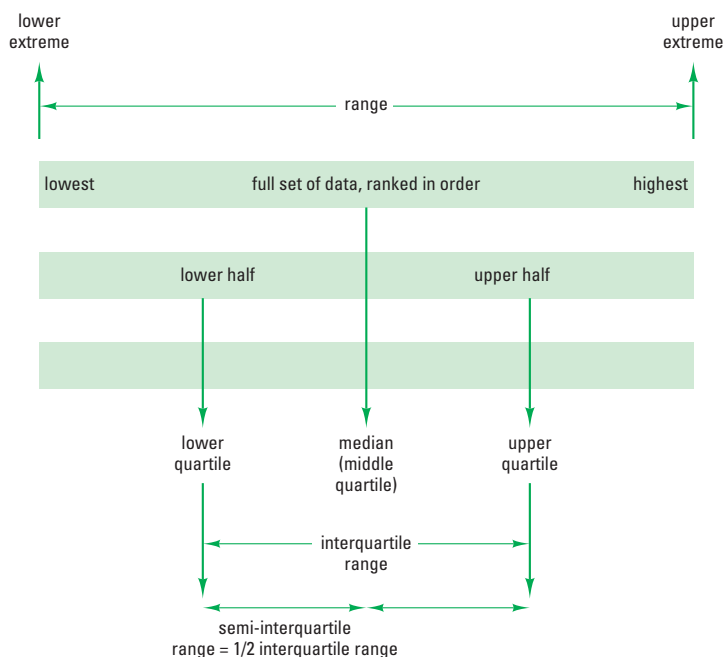


Fig. 76.6 Illustration of median, quartiles, range and semi-interquartile range.

Example In a sample of data with values 3, 7, 15, 8, 5, 10 and 4, the range is 12 (i.e. the difference between the highest value, 15, and the lowest value, 3).

1. Rank the observations in ascending order.
2. Find the values of the first and third quartiles.
3. Subtract the value of the first quartile from the value of the third.
4. Halve this number.

For data grouped in classes, the semi-interquartile range can only be estimated. Another disadvantage is that it takes no account of the shape of the distribution at its edges. This objection can be countered by using the so-called 'five-number summary' of a data set, which consists of the three quartiles and the two extreme values; you can present this on graphs as a box-and-whisker plot (see (Fig. 76.7) and it is particularly useful for summarising skewed frequency distributions. The corresponding 'six-number summary' includes the sample's size.

Variance and standard deviation

For symmetrical frequency distributions, an ideal measure of dispersion would take into account each value's deviation (=distance) from the mean and provide a measure of the average deviation from the mean. Two such statistics are the sample variance, which is the sum of the squared deviations of each data value from the mean 'the sum of squares' ($\sum (Y - \bar{Y})^2$) divided by $n - 1$ (where n is the sample size), and the sample standard deviation, which is the positive square root of the sample variance.

The variance (s^2) has units that are the square of the original units, while the standard deviation (s , or SD) is expressed in the original units, one reason s is often preferred as a measure of dispersion. Calculating s or s^2

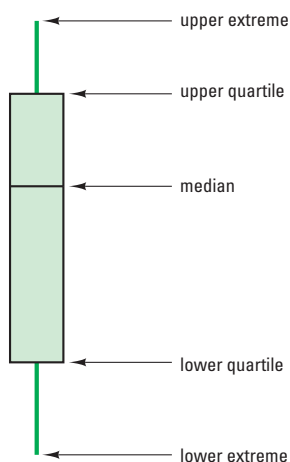


Fig. 76.7 A box-and-whisker plot, showing the 'five-number summary' of a sample as it might be used on a graph.

longhand is a tedious job and is best done with the help of a calculator or computer. If you do not have a calculator that calculates s for you, an alternative formula that simplifies calculations is:

$$s = +\sqrt{\frac{\sum Y^2 - (\sum Y)^2/n}{n-1}} \quad [76.1]$$

Using a calculator for statistics – make sure you understand how to enter individual data values and which keys will give the sample mean (usually shown as \bar{X} or \bar{x}) and sample standard deviation (often shown as σ_{n-1}). In general, you should not use the population standard deviation (usually shown as σ_n) unless you are working with all individuals in a population.

To calculate s using a calculator without a dedicated function:

1. Obtain $\sum Y$, square it, divide by n and store in memory.
2. Square individual Y values, add together to give $\sum Y^2$, then subtract memory value from this.
3. Divide this answer by $n - 1$.
4. Take the positive square root of this value.

Take care to retain significant figures, or you are likely to introduce errors in the final value of s . If continuous data have been grouped into classes, the class mid-values and their squares must be multiplied by the appropriate frequencies before summation. When data values are large, you can simplify longhand calculations by coding the data, for example, by subtracting a constant from each datum, and adding back the constant once the simplified calculations are complete (see Sokal and Rohlf, 2012).

Coefficient of variation

The coefficient of variation (CoV) is a dimensionless measure of dispersion relative to location that expresses the sample standard deviation, usually as a percentage of the sample mean, i.e.

$$\text{CoV} = 100s/\bar{Y}(\%) \quad [76.2]$$

You may find this statistic useful when comparing the relative dispersion of data sets with widely differing means or where different units have been used for the same or similar quantities.

A useful application of the CoV is to compare different analytical methods or procedures, so that you can decide which involves the least proportional error. For example, you might create a standard stock solution, then base your comparison of methods on the results from several subsamples analysed by each procedure. You may find it useful to use the CoV to compare the precision of your own results with those of a manufacturer, for example, when checking technique with a pipettor (pp. 143–4). The smaller the CoV, the more precise (repeatable) is the apparatus or technique (note: this does not mean that it is necessarily more *accurate*).

Measuring the precision of a sample mean

Most practical exercises are based on a limited number of individual data values (a sample) that are used to make inferences about the population from which they were drawn. For example, the haemoglobin content might be measured in blood samples from 100 adult females and used as an estimate of the adult female haemoglobin content, with the sample mean (\bar{Y}) and sample standard deviation (s) providing estimates of the true values of the underlying population mean (m) and the population standard deviation (s). The reliability of the sample mean as an estimate of the true (population) mean can be assessed by calculating a statistic termed the standard error of the sample mean (often abbreviated to standard error or SE), from:

$$\text{SE} = s/\sqrt{n} \quad [76.3]$$

Example Consider two methods of bioassay for a toxin in fresh water. For a given standard, Method A gives a mean result of = 50 'response units' with $s = 8$, while Method B gives a mean result of = 160 'response units' with $s = 18$. Which bioassay gives the more reproducible results? The answer can be found by calculating the CoV values, which are 16% and 11.25% respectively. Hence, Method B is the more precise, even though the absolute value of s is larger.

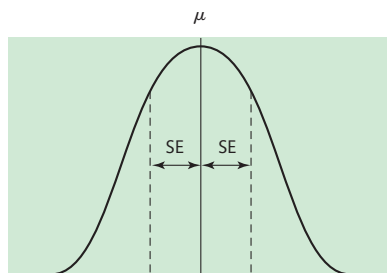


Fig. 76.8 Frequency distribution of sample means around the population mean (μ). Note that SE is equivalent to the standard deviation of the sample means, for sample size = n .

Strictly, the standard error is an estimate of the dispersion of repeated sample means around the true (population) value: if several samples were taken, each with the same number of data values (n), then you would find their means would cluster around the population mean (μ) with a standard deviation equal to SE, as shown in Fig. 76.8. Therefore, the *smaller* the SE, the more reliable the sample mean is likely to be as an estimate of the true value, since the underlying frequency distribution would be more tightly clustered around μ . At a practical level, eqn [76.3] shows that SE is directly affected by the dispersion of individual data values within the sample, as represented by the sample standard deviation (s). Perhaps more importantly, SE is inversely related to the *square root* of the number of data values (n). Therefore, if you wanted to increase the precision of a sample mean by a factor of 2 (i.e. to reduce SE by half), you would have to increase n by a factor of 2^2 (i.e. four-fold).

Summary descriptive statistics for the sample mean are often quoted as $\bar{Y} \pm \text{SE}(n)$, with the SE being given to one significant figure more than the mean. For example, summary statistics for the sample mean and standard error for the data shown in Box 76.1 would be quoted as 4.95 ± 0.280 ($n = 21$). You can use such information to carry out a t -test between two sample means (Box 77.1); the SE is also useful because it allows calculation of confidence limits for the sample mean.

Describing the shape of frequency distributions

Frequency distributions may differ in the following characteristics:

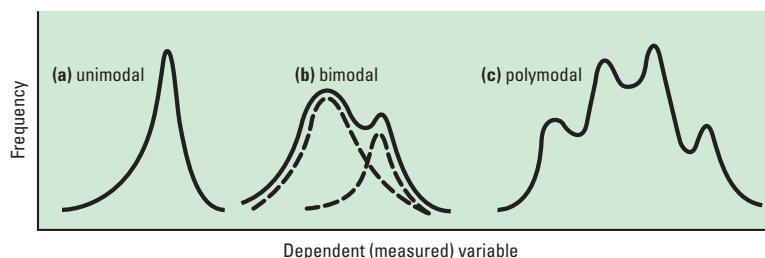
- **number of peaks**
- **skewness (asymmetry)**
- **kurtosis (pointedness).**

The shape of a frequency distribution of a small sample is affected by chance variation and may not be a fair reflection of the underlying population frequency distribution: you can check this by comparing repeated samples from the same population or by increasing the sample size. If the original shape were due to random events, it should not appear consistently in repeated samples and should become less obvious as sample size increases.

Genuinely bimodal or polymodal distributions may result from the combination of two or more unimodal distributions, indicating that more than one underlying population is being sampled (Fig. 76.9). An often-quoted example of a bimodal distribution is the height of adult humans (females and males combined).

A distribution is skewed if it is not symmetrical, a symptom being that the mean, median and mode are not equal (Fig. 76.3). Positive skewness

Fig. 76.9 Frequency distributions with different numbers of peaks. A unimodal distribution (a) may be symmetrical or asymmetrical. The dotted lines in (b) indicate how a bimodal distribution could arise from a combination of two underlying unimodal distributions. Note here how the term 'bimodal' is applied to any distribution with two major peaks – their frequencies do not have to be exactly the same.



Box 76.2 How to avoid errors when calculating simple arithmetic means

Mean	N
6	4
7	7
8	1

pH value	$[H^+]$ (mol L ⁻¹)
6	1×10^{-6}
7	1×10^{-7}
8	1×10^{-8}
mean	3.7×10^{-7}
$-\log_{10}$ mean	6.43

- 1. If you are working with means of samples that are themselves averaged, an error can arise if the samples are of different size.** For example, the arithmetic mean of the means in the table shown left is 7, but this does not take account of the different number of values to calculate each mean. The correct weighted mean is obtained by multiplying each mean by its sample size (n = 'weight') and dividing the sum of these values by the total number of observations, i.e. in the case shown, $(24 + 49 + 8)/12 = 6.75$.
- 2. When you are calculating a mean using ratios (e.g. percentages) for several groups of different sizes, the ratio for the combined total of all the groups is not the mean of the proportions for the individual groups.** For example, if 20 rats from a batch of 50 are male, this implies 40% are male. If 60 rats from a batch of 120 are male, this implies 50% are male. The mean percentage of males $(50 + 40)/2 = 45\%$ is not the percentage of males in the two groups combined, because there are $20 + 60 = 80$ males in a total of 170 rats = 47% (to the nearest integer).
- 3. If you are working with a measurement scale that is not linear, arithmetic means may give a false value.** For example, if three solutions had pH values 6, 7 and 8, the appropriate mean pH is not 7 because the pH scale is logarithmic. The definition of pH is $-\log_{10}[H^+]$, where $[H^+]$ is expressed in mol L⁻¹ ('molar'); therefore, to obtain the true mean, convert data into $[H^+]$ values (i.e. put them on a linear scale) by calculating $10^{(-\text{pH value})}$ as in the table shown left. Now calculate the mean of these values and convert the answer back into pH units. Thus, the appropriate answer is pH 6.43 rather than 7. Note that a similar procedure is necessary when calculating statistics of dispersion in such cases, so you will find these almost certainly asymmetric about the mean.

Mean values of log-transformed data are often termed geometric means – they are sometimes used in microbiology and in cell culture studies, where log-transformed values for cell density counts are averaged and plotted rather than using the raw data values. The use of geometric means in such circumstances serves to reduce the effects of outliers on the mean.

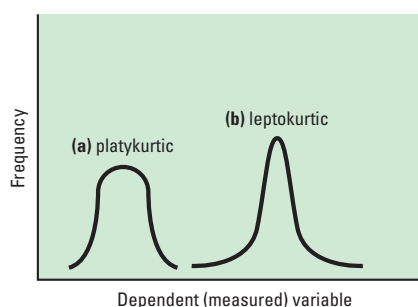


Fig. 76.10 Examples of the two types of kurtosis.

is where the longer 'tail' of the distribution occurs for higher values of the measured variable; negative skewness where the longer tail occurs for lower values. Some biological examples of characteristics distributed in a skewed fashion are volumes of plant protoplasts, insulin levels in human plasma and bacterial colony counts.

Kurtosis is the name given to the 'pointedness' of a frequency distribution. A platykurtic frequency distribution is one with a flattened peak, while a leptokurtic frequency distribution is one with a pointed peak (Fig. 76.10). While descriptive terms can be used, based on visual observation of the shape and direction of skew, the degree of skewness and kurtosis can be quantified and statistical tests exist to test the 'significance' of observed values (see Sokal and Rohlf, 2012), but the calculations required are complex and best done by computer.

Using software to calculate descriptive statistics

You can determine some descriptive statistics with advanced calculators, but for reasonably large data sets, spreadsheets may be more appropriate. These offer sophisticated statistical analysis functions, some examples of which are provided in Box 76.3. There are also many dedicated online statistical packages (for example, *Minitab*, *SYSTAT*, *SigmaXL* and *SPSS*) Note that correct interpretation of the output of any software program requires an understanding of the terminology used and the underlying process of calculation, and you may best gain this by working through one or more examples by hand before using these tools.

Box 76.3 How to use a spreadsheet to calculate descriptive statistics

Method 1: Using spreadsheet functions to generate the required statistics

Suppose you had obtained the following set of data, stored within an array (block of columns and rows) of cells (A2:L6) within a spreadsheet:

	A	B	C	D	E	F	G	H	I	J	K	L
1	My data set											
2	4	4	3	3	5	4	3	7	7	3	5	3
3	6	2	9	7	3	4	5	6	6	9	4	8
4	5	3	2	5	4	5	7	2	8	3	6	3
5	11	3	5	2	4	3	7	8	4	4	4	3
6	3	6	8	5	6	4	3	4	3	6	10	5

Taking Microsoft *Excel* as an example, the following functions could be used to extract information from the data set. Note that the precise syntax required may depend on the spreadsheet version used.

Descriptive statistic	Example of use of function ^{a, b}	Result for the above data set
Sample size <i>n</i>	=COUNT(A2:L6)	60
Mean	=AVERAGE(A2:L6) ^c	4.9
Median	=MEDIAN(A2:L6)	4.0
Mode	=MODE(A2:L6)	3
Upper quartile	=QUARTILE(A2:L6,3) ^d	6.0
Lower quartile	=QUARTILE(A2:L6,1)	3.0
Semi-interquartile range	=QUARTILE(A2:L6,3)-QUARTILE(A2:L6,1)	3.0
Upper extreme	=QUARTILE(A2:L6,4) or =MAX(A2:L6)	11
Lower extreme	=QUARTILE(A2:L6,0) or =MIN(A2:L6)	2
Range	=MAX(A2:L6)-MIN(A2:L6) ^e	9.0
Variance	=VAR(A2:L6)	4.464
Standard deviation	=STDEV(A2:L6)	2.113
Standard error	=STDEV(A2:L6)/(SQRT(COUNT(A2:L6))) ^f	0.273
Coefficient of variation	=100*STDEV(A2:L6)/AVERAGE(A2:L6)	43.12%

Notes:

^aTypically, for *Excel*, in an appropriate cell, you would, for example, select *Formulas > More Functions > Statistical > COUNT*, then select the input range and press return.

^bOther descriptive statistics can be calculated – these mirror those shown in Box 76.1, but for this specific data set.

^cThere is no 'MEAN' function in Microsoft *Excel*.

^dThe first argument within the brackets relates to the array of data, the second relates to the quartile required (consult the *Help on this function* feature for further information).

^eThere is no direct 'RANGE' function in Microsoft *Excel*.

^fThere is no direct 'STANDARD ERROR' function in Microsoft *Excel*. The SQRT function returns a square root and the COUNT function determines the number of filled data cells in the array.

(continued)

Box 76.3 (continued)**Method 2: Using the Tools > Data Analysis option in Excel**

This can automatically generate a table of descriptive statistics for the data array selected, although the data must be presented as a single row or column. This option might need to be installed for your network or personal computer before it is available to you (see Box 74.2, p. 571 for instructions). Having entered or rearranged your data into a row or column, the steps involved are as follows:

1. Select **Data > Data Analysis**.
2. From the **Data Analysis** box, select **Descriptive Statistics**.
3. Input your data location into the **Input Range** (left-click and hold down to highlight the column of data).
4. From the menu options, select **Summary Statistics** and **Confidence Level for Mean: 95%**.
5. When you click **OK** you should get a new worksheet, with descriptive statistics and confidence limits shown. Alternatively, at step 3, you can select an area of your current worksheet as a data output range (select an area away from any existing content as these cells would otherwise be overwritten by the descriptive statistics output table).
6. Change the format of the cells to show each number to an appropriate number of decimal places. You may also wish to make the columns wider so you can read their content.
7. For the data set shown above, the final output table should look as shown in Table 76.1.

Table 79.1 Descriptive statistics for a data set

Column1 ^{a, b}	
Mean	4.9
Standard error	0.27
Median	4.0
Mode	3
Standard deviation	2.113
Sample variance	4.464
Kurtosis	0.22
Skewness	0.86
Range	9.00
Minimum	2.0
Maximum	11.0
Sum	294
Count	60
Confidence level (95.0%)	0.55

Notes:

^aThese descriptive statistics are specified (and are automatically presented in this order) – any others required can be generated using Method 1.

^bA more descriptive heading can be added if desired – this is the default.

Notes: Spreadsheet commands illustrated here may vary among the various versions of Microsoft *Office* programs and for different spreadsheet products. Check the precise functions and syntax using the insert function menu option (f_x button in some versions of Excel).

Text references

Heath, D. (1995) *An Introduction to Experimental Design and Statistics for Biology*. UCL Press, London.

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Sources for further study

Ennos, R. and Johnson, M.L. (2018) *Statistical and Data Handling Skills in Biology*, 4th edn. Pearson, Harlow.

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Wardlaw, A.C. (2000) *Practical Statistics for Experimental Biologists*, 2nd edn. Wiley, New York.

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Urdan, T.C. (2016) *Statistics in Plain English*, 4th edn. Routledge, Abington.

STUDY EXERCISES

76.1 Choose appropriate descriptive statistics. For the following data and distribution types, state what statistics of location and dispersion (Figs 76.4 and 76.5) you should choose.

- pH of solutions, judged against a colour chart for pH.
- Number of bacterial colonies on replicate Petri plates of agar medium.
- Body Mass Index of male and female class members.

76.2 Practise calculating descriptive statistics. Using the data set given below, calculate the following statistics:

- range
- variance
- standard deviation
- coefficient of variation
- standard error.

Answers (b) to (e) should be given to four significant figures.

Set of data

9	6	7	5	7	7	8	6	5	5	7	8
5	8	7	7	6	7	8	6	5	7	7	6
3	6	8	9	9	6	7	8	5	6	5	5
8	8	7	5	6	5	8	6	7	5	7	6
5	6	7	8	7	6	7	7	8	8	9	4

76.3 Calculate and interpret standard errors. Two samples, A and B, gave the following descriptive statistics (measured in the same units): Sample A, mean = 16.2, standard deviation = 12.7, number of data values = 12; Sample B, mean = 13.2, standard deviation = 14.4, number of data values = 20. Which has the lower standard error in absolute terms and in proportion to the sample mean? (Express answers to three significant figures.)

76.4 Compute a mean value correctly. A researcher finds that the mean diameter of lichens on three exposed rocks designated A, B and C is 3.0, 2.5 and 2.0 mm respectively. He computes the mean lichen diameter as 2.5 mm, but forgets that the sample sizes were 24, 37 and 6 respectively. What is the true mean diameter of the lichens in this sample? (Answer to three significant figures.)

Answers to these study exercises are available at go.pearson.com/uk/he/resources

77 Selecting and interpreting statistical tests

Definitions

Hypothesis – this term is used in statistical contexts to describe a working theory about the data set(s) you are considering.

Hypothesis test – a statistical (i.e. mathematical) evaluation of the working theory.

Null hypothesis – a working assumption that there is no difference between the data sets you wish to compare (or no difference between a data set and a theoretical assumption about it, e.g. its distribution).

Alternative hypothesis – the opposing notion to the null hypothesis, i.e. that there is a difference between the data sets (or that the data set deviates from the theoretical prediction).

Significance – a measure of likelihood (probability) that the null hypothesis is correct.

This chapter outlines the philosophy and rationale of hypothesis-testing statistics and indicates the steps to be taken when you are choosing a test. It also discusses features and assumptions of some important tests. For details of how to carry out the tests, you should consult appropriate texts (for example, Wardlaw, 2000; Sokal and Rohlf, 2012). Most tests are now available in online statistical software (see p. 614) and many in spreadsheets (Chapter 72).

To carry out a statistical test:

1. **Decide what it is you wish to test** (create a null hypothesis and its alternative).
2. **Determine whether your data fit a standard distribution pattern.**
3. **Select a test and apply it to your data.**

Setting up a null hypothesis

Hypothesis-testing statistics are used to compare the properties of samples either with other samples or with some theory about them. For instance, you may be interested in whether two samples can be regarded as having different means, whether the counts of an organism in different quadrats can be regarded as randomly distributed, or whether property A of an organism is linearly related to property B.

KEY POINT You cannot use statistics to *prove* any hypothesis, but they can be used to assess *how likely* it is to be wrong.

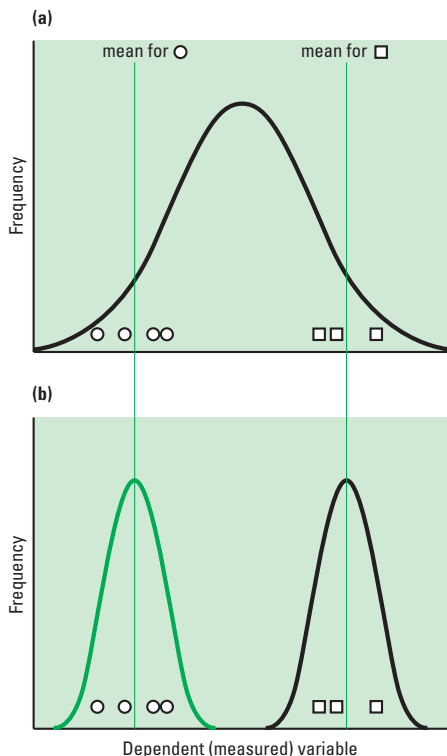


Fig. 77.1 Two explanations for the difference between two means. In case (a) the two samples happen by chance to have come from opposite ends of the same frequency distribution, i.e. there is no true difference between the samples. In case (b) the two samples come from different frequency distributions, i.e. there is a true difference between the samples. In both cases, the means of the two samples are the same.

Statistical testing operates in what at first seems a rather perverse manner. Suppose you think a treatment has an effect. The theory you actually test is that it has no effect; the test tells you how unlikely your data would be if this theory were true. This 'no effect' theory is the null hypothesis (NH). If your data are very improbable under the NH, then you may suppose it to be wrong, and this would support your original idea (the 'alternative hypothesis'). The concept can be illustrated by an example. Suppose two groups of subjects were treated in different ways, and you observed a difference in the mean value of the measured variable for the two groups. Can this be regarded as a 'true' difference? As Fig. 77.1 shows, it could have arisen in two ways:

1. **Because of the way the subjects were allocated to treatments**, i.e. all the subjects liable to have high values might, by chance, have been assigned to one group and those with low values to the other (Fig. 77.1(a)).
2. **Because of a genuine effect of the treatments**, i.e. each group came from a distinct frequency distribution (Fig. 77.1(b)).

A statistical test will indicate to you the probabilities of these options. The NH states that the two groups come from the same population (i.e. the treatment effects are negligible in the context of random variation). To test this, you calculate a test statistic from the data, and compare it with tabulated critical values giving the probability of obtaining the observed or

Understanding 'degrees of freedom' –

this depends on the number of values in the data set analysed, and the method of calculation depends on the statistical test being used. It relates to the number of observations that are free to vary before the remaining quantities for a data set can be determined.

Definition

Modulus – the absolute value of a number, e.g. modulus $-3.385 = 3.385$.

Quoting significance – the convention for quoting significance levels in text, tables and figures is as follows:

$P > 0.05$ = 'not significant' (or NS)
 $P \leq 0.05$ = 'significant' (or *)
 $P \leq 0.01$ = 'highly significant' (or **)
 $P \leq 0.001$ = 'very highly significant' (or ***)

Thus, you might refer to a difference in means as being 'highly significant ($P \leq 0.01$)'. For this reason, the word 'significant' in its everyday meaning of 'important' or 'notable' should be used with care in scientific writing. The asterisk coding is most often used as a short form in tables – if you use this coding, it will need to be explained in your table legend.

Choosing between parametric and non-parametric tests – plot your data graphically when determining whether they are suitable for parametric tests as this may save a lot of unnecessary effort later.

a more extreme result by chance (see Boxes 77.1 and 77.2). This probability is sometimes called the *significance* of the test.

Note that you must take into account the degrees of freedom (d.f.) when looking up critical values of most test statistics. The d.f. is related to the size(s) of the samples studied; formulae for calculating it depend on the test being used. Biologists normally use two-tailed tests, i.e. in an experiment, there is no expectation beforehand that a given treatment will have a positive or negative effect compared with the control (in a one-tailed test we test whether one particular treatment has a bigger effect than the other). Be sure to use critical values for the correct type of test. Consult your supervisor if unsure.

By convention, the critical probability for rejecting the NH is 5% (i.e. $P = 0.05$). This means you reject the NH if the observed result would have come up by chance a maximum of one time in twenty. If the modulus of the test statistic is less than or equal to the tabulated critical value for $P = 0.05$, then we accept the NH and the result is said to be 'not significant' (NS for short). If the modulus of the test statistic is greater than the tabulated value for $P = 0.05$, then we reject the NH in favour of the alternative hypothesis that the treatments had different effects and the result is 'statistically significant'.

Two types of error are possible when making a conclusion on the basis of a statistical test. The first occurs if you reject the NH when it is true (a so-called 'type I' error) and the second if you accept the NH when it is false (a so-called type II error). To limit the chance of the first type of error, choose a lower probability, for example $P = 0.01$, but note that the critical value of the test statistic increases when you do this and results in the probability of the second type of error increasing. The conventional significance levels given in statistical tables (usually 0.05, 0.01, 0.001) are somewhat arbitrary. Increasing use of statistical computer programs now allows the *actual* probability of obtaining the calculated value of the test statistic to be quoted (for example, $P = 0.037$).

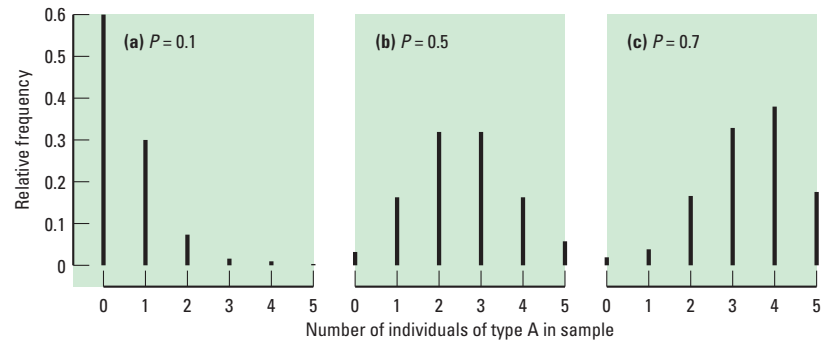
Note that if the NH is rejected, this does not tell you why: many alternative explanations may be possible. Also, it is important to distinguish between statistical significance and biological relevance: identifying a statistically significant difference between two samples does not mean that this will carry any biological importance. For example, the difference, though judged significant in a statistical test, may be small in numerical terms.

Comparing data with parametric distributions

The distribution pattern of a set of data values may be biologically relevant, and it is also of practical importance because it defines the type of statistical tests that you can use. A *parametric* test is one that makes particular assumptions about the mathematical nature of the population distribution from which the samples were taken. If these assumptions are not true, then the test is obviously invalid, even though it might give an answer of interest. A *non-parametric* test does not assume that the data fit a particular pattern, but it may assume some characteristics of their distributions. Used in appropriate circumstances, parametric tests are better able to distinguish between true but marginal differences between samples than their non-parametric equivalents (i.e. they have greater 'power').

The properties of the main distribution types found in biology are given below with both rules of thumb and more rigorous tests for deciding whether data fit these distributions.

Fig. 77.2 Examples of binomial frequency distributions with different probabilities. The distributions show the expected frequency of obtaining n individuals of type A in a sample of 5. Here P is the probability of an individual being type A rather than type B.



Binomial distributions

These apply to samples of any size from populations when data values occur independently in only two mutually exclusive classes (for example, possessing type A or type B characteristic, such as eye colour). They describe the probability of finding the different possible combinations of the characteristic for a specified sample size k (for example, out of 10 specimens, what is the chance of 8 being type A?). If p is the probability of the attribute being of type A and q the probability of it being type B, then the expected mean sample number of type A is kp and the standard deviation is \sqrt{kpq} . Expected frequencies can be calculated using mathematical expressions (see Sokal and Rohlf, 2012). Examples of the shapes of some binomial distributions are shown in Fig. 77.2. Note that they are symmetrical in shape for the special case $p = q = 0.5$ and the greater the disparity between p and q , the more skewed the distribution.

Some biological examples of data likely to be distributed in binomial fashion are: possession of two alleles for seed-coat morphology (for example, smooth and wrinkly); whether an organism is infected with a microbe or not; whether an animal is male or female. Binomial distributions are particularly useful for predicting gene segregation in Mendelian genetics (Chapter 65) and can be used for testing whether combinations of events have occurred more frequently than predicted (for example, more siblings being of the same sex than expected). To establish whether a set of data is distributed in binomial fashion: calculate expected frequencies from probability values obtained from theory or observation, then test against observed frequencies using a χ^2 test (p. 497) or a G test (see Wardlaw, 2000).

Poisson distributions

These apply to discrete characteristics that can assume low whole number values, such as counts of events occurring in area, volume or time. The events should be 'rare' in that the mean number observed should be a small proportion of the total that could possibly be found. Also, finding one count should not influence the probability of finding another. The shape of Poisson distributions is described by only one parameter, the mean number of events observed, and has the special characteristic that the variance is equal to the mean. The shape has a pronounced positive skewness at low mean counts, but becomes more and more symmetrical as the mean number of counts increases (Fig. 77.3).

Some examples of characteristics distributed in a Poisson fashion are: number of plants in a quadrat; number of microbes per unit volume

Tendency towards the Gaussian

distribution – under certain conditions, binomial and Poisson distributions can be treated as Gaussian:

- where samples from a binomial distribution are large (i.e. > 15) and p and q are close to 0.5
- for Poisson distributions, if the number of counts recorded in each outcome is greater than about 15.

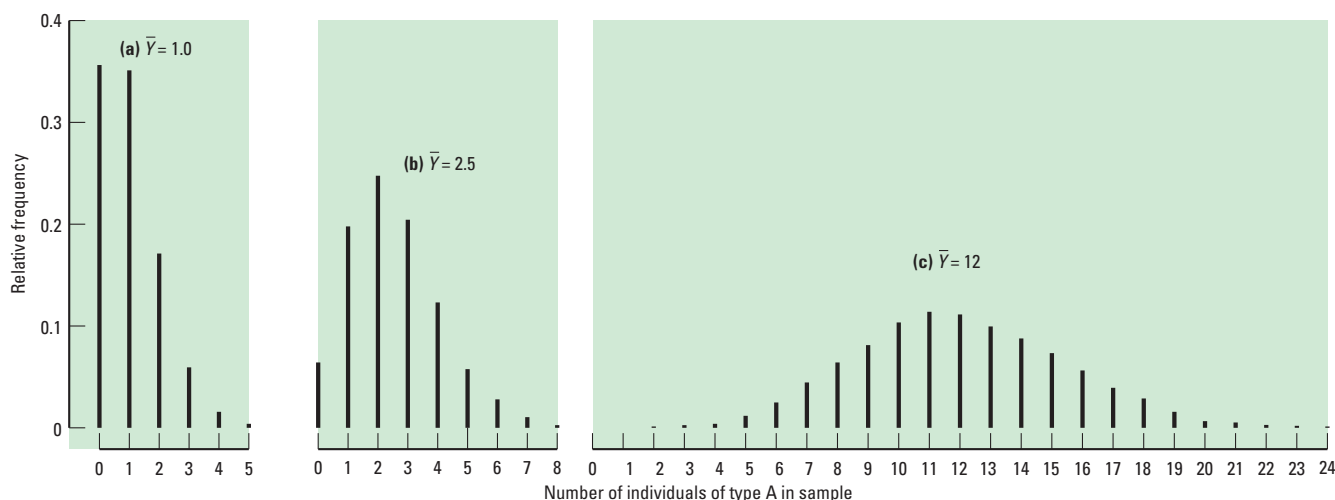


Fig. 77.3 Examples of Poisson frequency distributions differing in mean. The distributions are shown as line charts because the independent variable (events per sample) is discrete.

Definition

Coefficient of dispersion = s^2/\bar{Y} . This is an alternative measure of dispersion to the coefficient of variation (p. 596).

Understanding the terminology of distributions – ‘Gaussian’ distributions are those that follow a function defined by the mathematician Carl Gauss. These symmetrical bell-shaped distributions are also called ‘normal’ but this term can be confusing – it does not necessarily imply ‘normality’ in the sense of ‘typical’.

Definition

Homogeneous variance – uniform (but not necessarily identical) variance of the dependent variable across the range of the independent variable. The term homoscedastic is also used in this sense. The opposite of homogeneous is heterogeneous (= heteroscedastic).

of medium; number of animals parasitised per unit time; number of radioactive disintegrations per unit time. One of the main uses for the Poisson distribution is to quantify errors in count data such as estimates of cell densities in dilute suspensions (see p. 245). To decide whether data are Poisson distributed:

- Use the rule of thumb that if the coefficient of dispersion ≈ 1 , the distribution is likely to be Poisson.
- Calculate ‘expected’ frequencies from the equation for the Poisson distribution and compare with actual values using a χ^2 test or a *G*-test.

It is sometimes of interest to show that data are *not* distributed in a Poisson fashion, for example, the distribution of parasite larvae in hosts, or the frequency of extinction events in geological time. If $s^2/\bar{Y} > 1$, the data are ‘clumped’ and occur together more than would be expected by chance; if $s^2/\bar{Y} < 1$, the data are ‘repulsed’ and occur together less frequently than would be expected by chance.

Gaussian distributions (‘normal’ distributions)

These occur when random events act to produce variability in a continuous characteristic (quantitative variable). This situation occurs frequently in biology, so Gaussian distributions are very useful and much used. The bell-like shape of these distributions is specified by the population mean and standard deviation (Fig. 77.4): it is symmetrical and configured such that 68.27% of the data will lie within ± 1 standard deviation of the mean, 95.45% within ± 2 standard deviations of the mean, and 99.73% within ± 3 standard deviations of the mean.

Some biological examples of data likely to be distributed in a Gaussian fashion are: fresh weight of plants of the same age; linear dimensions of bacterial cells; height of either adult female or male humans. To test whether data fit a Gaussian distribution, you can:

- Use the rule of thumb that the distribution should be symmetrical and that nearly all the data should fall within $\pm 3s$ of the mean and about two-thirds within $\pm 1s$ of the mean.

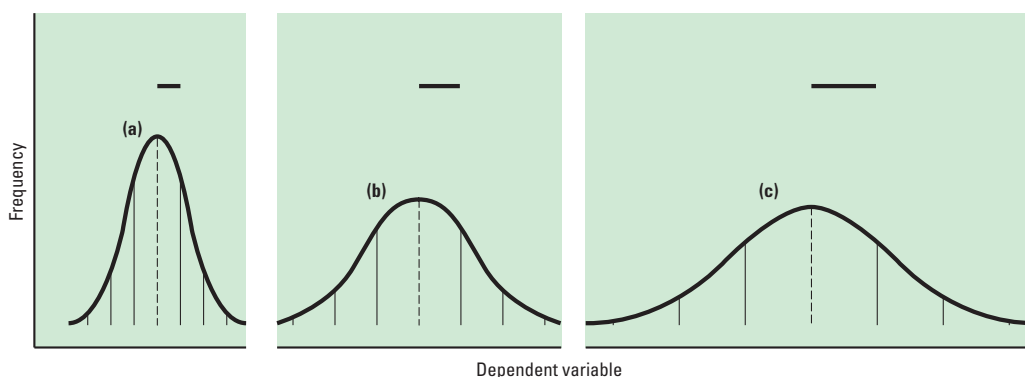


Fig. 77.4 Examples of Gaussian frequency distributions differing in mean and standard deviation. The horizontal bars represent population standard deviations for the curves, increasing from (a) to (c). Vertical dashed lines are population means, while vertical solid lines show positions of values ± 1 , 2 and 3 standard deviations from the means.

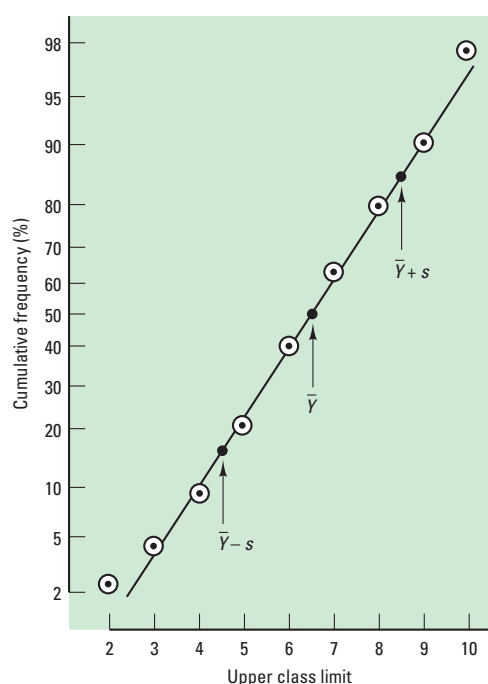


Fig. 77.5 Example of a Gaussian probability plot. The plotted points are from a small data set where the mean $\bar{Y} = 6.93$ and the standard deviation $s = 1.895$. Note that values corresponding to 0% and 100% cumulative frequency cannot be used. The straight line is that predicted for a Gaussian distribution with $\bar{Y} = 6.93$ and $s = 1.895$. This is plotted by calculating the expected positions of points for $\bar{Y} \pm s$. Since 68.3% of the distribution falls within these bounds, the relevant points on the cumulative frequency scale are $50 \pm 34.15\%$; thus this line was drawn using the points (4.495, 15.85) and (8.285, 84.15) as indicated on the plot.

- **Plot the distribution on ‘normal probability’ graph paper.** If the distribution is Gaussian, the data will tend to follow a straight line (see Fig. 77.5). Deviations from linearity reveal skewness and/or kurtosis (see p. 598), the significance of which can be tested statistically (see Sokal and Rohlf, 2012).
- **Use suitable statistical software to generate predicted Gaussian curves** from the \bar{Y} and s values of your sample(s). These can be compared visually with the actual distribution of data and can be used to give ‘expected’ values for a χ^2 test or a G -test.

The wide availability of tests based on the Gaussian distribution and their relative simplicity means you may wish to transform your data to make them more like a Gaussian distribution. Table 77.1 provides transformations that might be applied (see also Fig. 74.3). The transformed data should be tested to see if they are Gaussian in nature as described above before proceeding – do not forget that you may need to check that transformed variances are homogeneous for certain tests (see below).

A very important theorem in statistics, the Central Limit Theorem, states that as sample size increases, the distribution of a series of means from *any* type of frequency distribution will become distributed in a Gaussian fashion. This fact can be used to devise an experimental or sampling strategy that ensures that data follow a Gaussian pattern, i.e. by using means of

Table 77.1 Suggested transformations altering different types of frequency distributions to the Gaussian type. To use, modify data by the formula shown; then examine effects with the tests described on pp. 549–51

Type of data; distribution suspected	Suggested transformation(s)
Proportions (including percentages); binomial	arcsine \sqrt{x} (also called the angular transformation)
Scores; Poisson	\sqrt{x} or $\sqrt{(x + 1/2)}$ if zero values present
Measurements; negatively skewed	x^2 , x^3 , x^4 , etc. (in order of increasing strength)
Measurements; positively skewed	$1/\sqrt{x}$, \sqrt{x} , $\ln X$, $1/x$ (in order of increasing strength)

samples as if they were primary data. Note that the samples should be truly independent and not sub-samples.

Choosing a suitable statistical test

Comparing location (for example, means)

If you can assume that your data are distributed in a Gaussian fashion, the main test for comparing two means from independent samples is the t -test (see Boxes 77.1–77.3, and Table 77.2). This assumes that the variances of the data sets are homogeneous. Tests based on the t -distribution are also available for two other situations, (i) comparing means of paired data and (ii) comparing a sample mean with a chosen value.

When comparing means of two or more samples, analysis of variance (ANOVA) is a very useful technique. This method also assumes data have a Gaussian distribution and that the variances of the samples are homogeneous. The samples must also be independent (for example, not subsamples). The test statistic calculated is denoted F and it has two different degrees of freedom related to the number of means tested and the pooled number of replicates per mean (Box 77.1). The nested types of ANOVA are useful for letting you know the relative importance of different sources of variability in your data. Two-way and multi-way ANOVAs are useful for studying interactions between treatments.

Table 77.2 Critical values of Student's t statistic (for two-tailed tests). Reject the null hypothesis at probability P if your calculated t value equals or exceeds the value shown for the appropriate degree of freedom = $(n_1 - 1) + (n_2 - 1)$

Degrees of freedom	Critical values for $P = 0.05$	Critical values for $P = 0.01$	Critical values for $P = 0.001$
1	12.71	63.66	636.62
2	4.30	9.92	31.60
3	3.18	5.84	12.94
4	2.78	4.60	8.61
5	2.57	4.03	6.86
6	2.45	3.71	5.96
7	2.36	3.50	5.40
8	2.31	3.36	5.04
9	2.26	3.25	4.78
10	2.23	3.17	4.59
12	2.18	3.06	4.32
14	2.14	2.98	4.14
16	2.12	2.92	4.02
20	2.09	2.85	3.85
25	2.06	2.79	3.72
30	2.04	2.75	3.65
40	2.02	2.70	3.55
60	2.00	2.66	3.46
120	1.98	2.62	3.37
∞	1.96	2.58	3.29

Box 77.1 How to carry out a *t*-test – principles and practice

The *t*-test was devised by a statistician who used the pen name 'Student', so you may see it referred to as Student's *t*-test. It is used when you wish to decide whether two samples come from the same population or from different ones (Fig. 77.1). The samples might have been obtained by selective observation or by applying two different treatments to an originally homogeneous population (Chapter 29).

The null hypothesis (NH) is that the two groups can be represented as samples from the same overlying population (Fig. 77.1(a)). If, as a result of the test, you accept this hypothesis, you can say that there is no significant difference between the group means.

The alternative hypothesis is that the two groups come from different populations (Fig. 77.1(b)). By rejecting the NH as a result of the test, you can accept the alternative hypothesis and say that there is a significant difference between the sample means, or, if an experiment were carried out, that the two treatments affected the samples differently.

How can you decide between these two hypotheses? On the basis of certain assumptions (see below), and some relatively simple calculations, you can work out the probability that the samples came from the same population. If this probability is very low, then you can reasonably reject the NH in favour of the alternative hypothesis, and if it is high, you will accept the NH.

To find out the probability that the observed difference between sample means arose by chance, you must first calculate a '*t* value' for the two samples in question. Some computer programs (for example, *Minitab*) provide this probability as part of the output, otherwise you can look up statistical tables (e.g. Table 77.2). These tables show 'critical values' – the borders between probability levels. If your value of *t* equals or exceeds the critical value for probability *P*, you can reject the NH at this probability ('level of significance').

Note that:

- for a given difference in the means of the two samples, the value of *t* will get larger the smaller the scatter within each data set; and
- for a given scatter of the data, the value of *t* will get larger the greater the difference between the means.

So, at what probability should you reject the NH? Normally, the threshold is arbitrarily set at 5% – you quite often see descriptions like 'the sample means were significantly different ($P < 0.05$)'. At this 'significance level' there is still up to a 5% chance of the *t* value arising by chance, so about one in twenty times, on average, the conclusion will be wrong. If *P* turns out to be lower, then this kind of error is much less likely.

Tabulated probability levels are generally given for 5, 1 and 0.1% significance levels (see Table 77.2). Note that this table is designed for 'two-tailed' tests, i.e. where the treatment or sampling strategy could have resulted in either an increase or a decrease in the measured values. These are the most likely situations you will deal with in biology.

Examine Table 77.2 and note the following:

- The larger the size of the samples (i.e. the greater the 'degrees of freedom'), the smaller *t* needs to be to exceed the critical value at a given significance level.
- The lower the probability, the greater *t* needs to be to exceed the critical value.

The mechanics of the test

A calculator that can work out means and standard deviations is helpful.

1. **Work out the sample means \bar{Y}_1 and \bar{Y}_2 and calculate the difference between them.**
2. **Work out the sample standard deviations s_1 and s_2 .** (NB if your calculator offers a choice, choose the ' $n - 1$ ' option for calculating *s* – see pp. 595–6).
3. **Work out the sample standard errors $SE = s_1/\sqrt{n_1}$ and $SE_2 = s_2/\sqrt{n_2}$; now square each, add the squares together, then take the positive square root of this** (n_1 and n_2 are the respective sample sizes, which may, or may not, be equal).
4. **Calculate *t* from the formula:**

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{\sqrt{[(SE_1)^2] + [(SE_2)^2]}} \quad [77.1]$$

The value of *t* can be negative or positive, depending on the values of the means; this does not matter and you should compare the modulus (absolute value) of *t* with the values in tables.

5. **Work out the degrees of freedom $= (n_1 - 1) + (n_2 - 1)$.**
6. **Compare the *t* value with the appropriate critical value (see e.g. Table 77.2) and decide on the significance of your findings** (see p. 603).

Boxes 77.2 and 77.3 provide worked examples – use these to check that you understand the above procedures.

Assumptions that must be met before using the test

The most important assumptions are:

- The two samples are independent and randomly drawn (or if not, drawn in a way that does not create bias). The test assumes that the samples are quite large.
- The underlying distribution of each sample is Gaussian. This can be tested with a special statistical test, but a rule of thumb is that a frequency distribution of the data should be (a) symmetrical about the mean and (b) nearly all of the data should be within 3 standard deviations of the mean and about two-thirds within 1 standard deviation of the mean (see p. 607).
- The two samples should have uniform variances. This again can be tested (by an *F*-test), but may be obvious from inspection of the two standard deviations.

Box 77.2 How to carry out a *t*-test – a worked example

Suppose the following data were obtained in an experiment (the units are not relevant):

Control: 6.6, 5.5, 6.8, 5.8, 6.1, 5.9

Treatment: 6.3, 7.2, 6.5, 7.1, 7.5, 7.3

Using the steps outlined in Box 77.1, the following values are obtained (denoting control with subscript 1, treatment with subscript 2):

1. $\bar{Y}_1 = 6.1167$; $\bar{Y}_2 = 6.9833$; difference between means = $\bar{Y}_1 - \bar{Y}_2 = -0.8666$

2. $s_1 = 0.49565$; $s_2 = 0.47504$

3. $SE_1 = 0.49565/2.44949 = 0.202348$
 $SE_2 = 0.47504/2.44949 = 0.193934$

$$4. t = \frac{-0.8666}{\sqrt{(0.202348^2 + 0.193934^2)}} = \frac{-0.8666}{0.280277} = -3.09$$

$$5. d.f = (5 + 5) = 10$$

6. Looking at Table 77.2, we see that the modulus of this *t* value exceeds the tabulated value for $P = 0.05$ at 10 degrees of freedom ($=2.23$). We therefore reject the H_0 , and conclude that the means are different at the 5% level of significance. If the modulus of *t* had been ≤ 2.23 , we would have accepted the H_0 . If the modulus of *t* had been >3.17 , we could have concluded that the means are different at the 1% level of significance.

Checking the assumptions of a test – always make sure you understand the assumptions of a test. If necessary, test them before using the test.

For data satisfying the ANOVA requirements, the least significant difference (LSD) is useful for making planned comparisons among several means (see Sokal and Rohlf, 2012). Any two means that differ by more than the LSD will be significantly different. The LSD is useful for showing on graphs.

The chief non-parametric tests for comparing the locations of two samples are the Mann–Whitney *U*-test and the Kolmogorov–Smirnov test. The former assumes that the frequency distributions of the samples are similar, whereas the latter makes no such assumption. In both cases the sample's size must be ≥ 4 and for the Kolmogorov–Smirnov test the samples must have equal sizes. In the Kolmogorov–Smirnov test, significant differences found with the test could be due to differences in location or shape of the distribution, or both.

Suitable non-parametric comparisons of location for paired data (sample size ≥ 6) include Wilcoxon's signed rank test, which is used for quantitative data and assumes that the distributions have similar shape. Dixon and Mood's sign test can be used for paired data scores where one variable is recorded as 'greater than' or 'better than' the other.

Non-parametric comparisons of location for three or more samples include the Kruskal–Wallis *H*-test. Here, the number of samples is without limit and they can be unequal in size, but again the underlying distributions are assumed to be similar. The Friedman *S*-test operates with a maximum of five samples and data must conform to a randomised block design. The underlying distributions of the samples are assumed to be similar.

Comparing dispersions (for example, variances)

If you wish to compare the variances of two sets of data that are distributed in a Gaussian fashion, use the *F*-test. For comparing more than two samples, it may be sufficient to use the F_{\max} -test, on the highest and lowest variances. The Scheffé–Box (log-ANOVA) test is recommended for testing the significance of differences between several variances. Non-parametric tests exist but are not widely available: you may need to transform the data and use a test based on the Gaussian distribution.

Confidence limits for statistics other than the mean – consult an advanced statistical text (e.g. Sokal and Rohlf, 2012) if you wish to indicate the reliability of estimates of e.g. population variances.

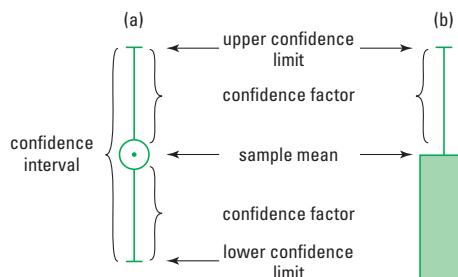


Fig. 77.6 Graphical representation of confidence limits as 'error bars' for (a) a sample mean in a plotted curve, where both upper and lower limits are shown; and (b) a sample mean in a histogram, where, by convention, only the upper value is shown. For data that are assumed to be symmetrically distributed, such representations are often used in preference to the 'box and whisker' plot shown in Fig. 76.7. Note that SE is an alternative way of representing sample imprecision/error (for example, Fig. 72.1).

Determining whether frequency observations fit theoretical expectation

The χ^2 test (Box 61.2) is useful for tests of 'goodness of fit', for example, comparing expected and observed progeny frequencies in genetical experiments or comparing observed frequency distributions with some theoretical function. One limitation is that simple formulae for calculating χ^2 assume that no expected number is less than 5. The *G*-test (2I-test) is used in similar circumstances.

Comparing proportion data

When comparing proportions between two small groups (for example, whether 3/10 is significantly different from 5/10), you can use probability tables such as those of Finney *et al.* (1963) or calculate probabilities from formulae; however, this can be tedious for large sample sizes. Certain proportions can be transformed so that their expected distribution becomes Gaussian.

Placing confidence limits on an estimate of a population parameter

On many occasions, a sample statistic is used to provide an estimate of a population parameter, and it is often useful to indicate the reliability of such an estimate. This can be done by putting confidence limits on the sample statistic, i.e. by specifying an interval around the statistic within which you are confident that the true value (the population parameter) is likely to fall, at a specified level of probability. The most common application is to place confidence limits on the mean of a sample taken from a Gaussian population of data values. In practice, you determine a confidence factor for a particular level of probability, then add and subtract this factor from the sample mean (\bar{Y}) to give the upper confidence limit and lower confidence limit respectively. Calculate these as:

$$\begin{aligned} \bar{Y} + (t_{P[n-1]} \times SE) & \text{ for the upper limit and} \\ \bar{Y} - (t_{P[n-1]} \times SE) & \text{ for the lower limit} \end{aligned} \quad [77.2]$$

where $t_{P[n-1]}$ is the tabulated critical value of Student's *t* statistic for a two-tailed test with $n - 1$ degrees of freedom at a specified probability level (*P*) and SE is the standard error of the sample mean (pp. 596–7). The 95% confidence limits (i.e. $P = 0.05$) tell you that, on average, 95 times out of 100 the interval between the upper and lower limits will contain the true (population) value. Confidence limits are often shown as 'error bars' when individual sample means are plotted in graphical form. Figure 77.6 illustrates how this is applied to plotted curves and histograms (note that this can be carried out for data series within a Microsoft *Excel* graph (chart) using the *Layout > Error Bars* commands).

Correlation and regression

These methods are used when you wish to test the relationship between data values for two variables. Correlation is used to measure the extent to which changes in the two sets of data values occur together in a linear manner (i.e. are co-related). If one variable can be assumed to be dependent upon the other (i.e. a change in *X* causes a particular change in *Y*), then regression techniques can be used to provide a mathematical description of the underlying relationship between the variables, for example, to find

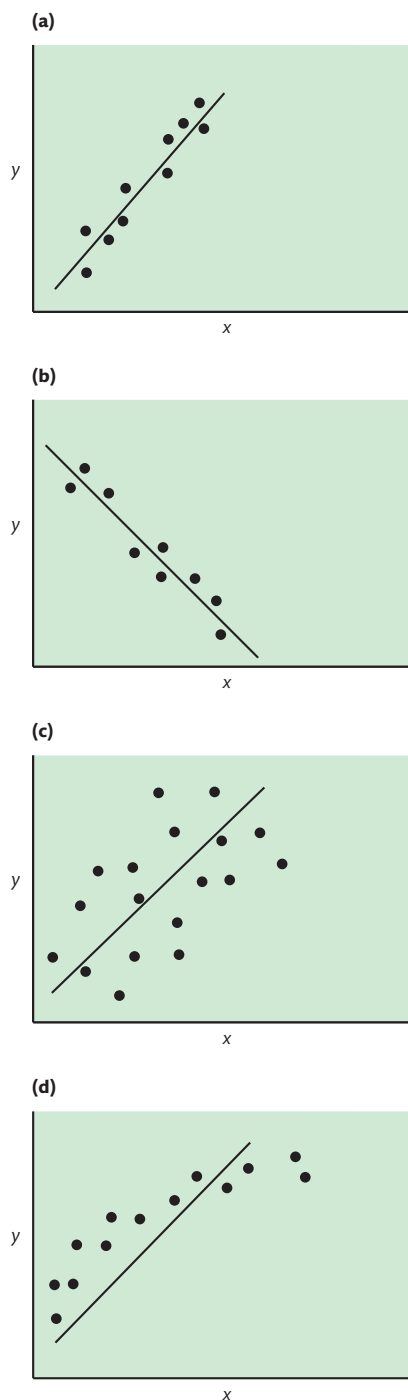


Fig. 77.7 Examples of correlation. The linear regression line is shown. In (a) and (b), the correlation between x and y is good: for (a) there is a positive correlation and the correlation coefficient, r , would be close to 1; for (b) there is a negative correlation and the correlation coefficient would be close to -1 . In (c) there is a weak positive correlation and r would be close to 0. In (d) the correlation coefficient may be quite large, but the choice of linear regression is clearly inappropriate.

a line of best fit for a data series. If there is no a priori reason to assume dependency, then correlation methods alone are appropriate.

A correlation coefficient measures the strength of the linear relationship between two variables, but does not describe the relationship. The coefficient is expressed as a number between -1 and $+1$: a positive coefficient indicates a direct relationship, where the two variables change in the same direction, while a negative coefficient indicates an inverse relationship, where one variable decreases as the other increases (Fig. 77.7). The nearer the coefficient is to -1 or $+1$, the stronger the linear relationship between the variables, i.e. the less ‘scatter’ there would be about a straight line of best fit (note that this does *not* necessarily imply that one variable is dependent upon the other). A coefficient of 0 implies that the two variables show no linear association and therefore the closer the correlation coefficient is to zero, the weaker the linear relationship. The importance of graphing data is shown by the case illustrated in Fig. 77.7(d).

Pearson’s product moment correlation coefficient (r) is the most commonly used statistic for testing correlations. The test is valid only if both variables are distributed in a Gaussian manner. Statistical tests can be used to decide whether the correlation is significant, for example using a one-sample t -test to see whether r is significantly different from zero, based on the equation:

$$t = r \div \sqrt{[(1 - r^2) \div (n - 2)]} \text{ at } n - 2 \text{ degrees of freedom, [77.3]}$$

where n is the number of paired observations. If one or both variables do not follow a Gaussian distribution, then you should calculate an alternative non-parametric coefficient, for example, Spearman’s coefficient of rank correlation (r_s) or Kendall’s coefficient of rank correlation (τ). These require the two sets of data to be ranked separately, and the calculation can be complex if there are tied (equal) ranks. Spearman’s coefficient is said to be better if there is any uncertainty about the reliability of closely ranked data values.

If underlying theory or empirical graphical analysis indicate a linear relationship between a dependent and an independent variable, then you can use linear regression to estimate the mathematical equation that links the two variables. Model I linear regression is the standard approach, and is available within general-purpose software programs such as Microsoft *Excel* (Box 77.3), and on some scientific calculators. It is suitable for experiments where a dependent variable Y varies with an *error-free* independent variable X in accordance with the relationship $Y = a + bX + e_Y$, where e_Y represents the residual (error) variability in the Y variable. For example, this relationship might apply in a laboratory procedure where you have carefully controlled the independent variable and the X values can be assumed to have zero error (for example, in a calibration curve, see Chapter 43, or in a time course experiment where measurements are made at exact time points). The regression analysis gives estimates for a and b (equivalent to the slope and intercept of the line of best fit): software programs usually provide additional features, for example residual values for Y (e_Y), estimated errors for a and b , predicted values of Y along with graphical plots of the line of best fit (the trend line) and the residual values. For the model to be valid, the residual (error) values should follow a Gaussian distribution around the trend line and their variance should be uniform (homogeneous), i.e. there should be a similar scatter of data points around the trend line along the x -axis (independent variable).

Box 77.3 How to use a spreadsheet to calculate hypothesis-testing statistics – an example

Presented below are three examples of the use of Microsoft *Excel* to investigate hypotheses about specific data sets. In each case, there is a brief description of the problem; a table showing the data analysed; an outline of the commands (for Microsoft *Excel*) used to carry out the analysis and an annotated table of results from the spreadsheet.

Example 1: A *t*-test

As part of a project, a student applied a chemical treatment to a series of flasks containing fungal cultures with nutrient solution. An otherwise similar set of control flasks received no chemical treatment. After three weeks' growth, she measured the wet mass of the filtered cultures:

Wet mass of samples (g)

Replicate	1	2	3	4	5	6	7	8	Mean	Variance
Treated with ZH52	2.342	2.256	2.521	2.523	2.943	2.481	2.601	2.449	2.515	0.042
Control	2.658	2.791	2.731	2.402	3.041	2.668	2.823	2.509	2.703	0.038

The student proposed the null hypothesis that there was no difference between the two means and tested this using a *t*-test, as she had evidence from other studies that the fungal masses of replicate flasks were distributed in a Gaussian fashion. She also established, by calculation, that the assumption that the populations had homogeneous variances was likely to be valid. She selected the option *t*-Test: Two-Sample Assuming Equal Variances, then entered data ranges as above and then 0 for *Hypothesized Mean Difference* and 0.05 for *Alpha* (= *P*). She selected a suitable output option and then OK. This returned a table of data as shown below.

***t*-test: Two-sample assuming equal variances**

	Variable 1	Variable 2
Mean	2.515	2.703
Variance	0.042	0.038
Observations	8	8
Pooled variance	0.040	
Hypothesised mean difference	0	
d.f.	14	
<i>t</i> Stat	−1.881	
<i>P</i> (T ≤ t) one-tail	0.040	
<i>t</i> Critical one-tail	1.801	
<i>P</i> (T ≤ t) two-tail	0.081	
<i>t</i> Critical two-tail	2.145	

The value of *t* obtained was −1.881 (row 7, '*t* stat') and the probability of obtaining this value for a two-tailed test (row 10) was 0.081 (or 8.1%), so the student was able to accept the null hypothesis and conclude that ZH52 had no significant effect on fungal growth in these circumstances.

Example 2: An ANOVA test

A biochemist made six replicate measurements of four different batches (A–D) of alcohol dehydrogenase, obtaining the following data:

Alcohol dehydrogenase activity (U L^{−1})

Batch/ Replicate	1	2	3	4	5	6	Mean	Variance
A	0.562	0.541	0.576	0.545	0.542	0.551	0.552833	0.000189
B	0.531	0.557	0.537	0.521	0.559	0.538	0.540500	0.000221
C	0.572	0.568	0.551	0.549	0.564	0.559	0.560500	0.000085
D	0.532	0.548	0.541	0.538	0.547	0.536	0.540333	0.000039

The biochemist wanted to know whether the observed differences were statistically significant, so he carried out an ANOVA test, assuming the samples had a Gaussian distribution and the variances in the three populations were homogeneous. He selected *Anova: Single Factor*, then entered the data ranges, selected *Grouped By: Rows* and set *Alpha* to 0.05 (= *P*). He selected a suitable output option and then OK. This returned a table of data as shown below.

ANOVA: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
A	6	3.317	0.552833	0.000189
B	6	3.243	0.5405	0.000221
C	6	3.363	0.5605	8.51E-05
D	6	3.242	0.540333	3.95E-05

ANOVA

Source of variation	SS	d.f.	MS	F	P-value	F crit
Between groups	0.001761	3	0.000587	4.397856	0.015669	3.098391
Within groups	0.002669	20	0.000133			
Total	0.00443	23				

The *F* value calculated was 4.397856. This comfortably exceeds the stated critical value (*F*_{crit}) of 3.098391, and the probability of obtaining this result by chance (*P*-value) was calculated as 0.015669 (1.57% to three significant figures); hence the biochemist was able to reject the null hypothesis and conclude that there was a significant difference in average enzyme activity between the four batches, since *P* < 0.05. Such a finding might lead on to an investigation into why there was batch variation, e.g. had they been stored differently?

(continued)

Box 77.3 (continued)**Example 3: Testing the significance of a correlation**

A researcher wanted to know whether there was any correlation between the levels of tar and nicotine in cigarettes. The researcher made measurements of both constituents for 10 different brands and obtained the following results.

Tar and nicotine content (mg g⁻¹) of cigarettes

Brand	Tar	Nicotine
1	12.6	0.92
2	8.5	0.58
3	15.7	1.14
4	32.5	2.16
5	14.5	1.03
6	3.1	0.17
7	18.9	1.32
8	7.7	0.98
9	10.7	1.13
10	6.2	0.55

The researcher assumed that both variables were normally distributed and used the Microsoft *Excel* function *PEARSON* (*array1*, *array2*) to obtain a value of +0.950 260 385 for the Pearson's product moment correlation coefficient *r*, specifying the tar content data as *array1* and the nicotine content data as *array2* (the *Excel* *CORREL* function can also be used to carry out the same task). The researcher then used a spreadsheet to calculate the *t* statistic for this *r* value, using the formula for Eqn [74.3]. The calculated value of *t* was 8.6296, with 8 degrees of freedom. The critical value from tables (e.g. Table 74.2) at *P* = 0.001 is 5.04, so the researcher concluded that there was a very highly significant positive correlation between the two constituents.

Spreadsheet commands illustrated here may vary among the various versions of Microsoft *Office* programs and for different spreadsheet products. Check the precise functions and syntax using the 'Insert function' menu option (fX or 'Tell me' button).

Using more advanced types of regression – these include:

- Model II linear regression, which applies to situations where a dependent variable *Y* varies with an independent variable *X*, and where both variables may have error terms associated with them.
- Multiple regression, which applies when there is a relationship between a dependent variable and two or more independent variables.
- Non-linear regression, which extends the principles of linear regression to a wide range of functions. Technically, this method is more appropriate than transforming data to allow linear regression.

Advanced statistics books should be consulted for details of these methods, which may be offered by some statistical computer programs.

If the relationship is not linear, try a transformation (see pp. 549–51). For example, this is commonly done in analysis of enzyme kinetics (see Fig. 62.4). However, you should be aware that the transformation of data to give a straight line can lead to errors when carrying out linear regression analysis: take care to ensure that (a) the assumptions listed in the previous paragraph are valid for your transformed data set and (b) the data points are evenly distributed throughout the range of the independent variable. If these criteria cannot be met, non-linear regression may be a better approach, but for this you will require a suitable software, for example, *GraphPad Prism*.

The strength of the relationship between *Y* and *X* in Model I linear regression is best estimated by the coefficient of determination (*r*² or *R*²), which is equivalent to the square of the Pearson correlation coefficient. The coefficient of determination varies between 0 and +1 and provides a measure of the goodness of fit of the *Y* data to the regression line: the closer the value is to 1, the better the fit. In effect, *r*² represents the fraction of the variance in *Y* that can be accounted for by the regression equation. Conversely, if you subtract this value from 1, you will obtain the residual (error) component, i.e. the fraction of the variance in *Y* that cannot be explained by the line of best fit. Multiplying the values by 100 allows you to express these fractions in percentage terms.

Example If a regression analysis gives a value for r^2 of 0.75 (i.e. $r = 0.84$), then 75% of the variance in Y can be explained by the trend line, with $1 - r^2 = 0.25$ (25%) remaining as unexplained (residual) variation.

Using spreadsheets for statistical tests – the hypothesis-testing statistical functions are usually reasonably powerful (e.g. t -test, ANOVA, regression) and they often return the *probability* (P) of obtaining the test statistic when the null hypothesis is true, so there may be no need to refer to statistical tables.

Using software to calculate hypothesis-testing statistics

As with the calculation of descriptive statistics (Chapter 76), you can use specialist statistical packages such as *SPSS* and *MINITAB* to simplify the calculation of hypothesis-testing statistics. The correct use of the software and interpretation of the output requires an understanding of relevant terminology and of the fundamental principles governing the test, which is probably best obtained by working through one or more examples by hand before using these tools (for example, Box 65.3, Box 77.2). Spreadsheets offer increasingly sophisticated statistical analysis functions, three examples of which are provided in Box 77.3.

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STUDY EXERCISES

77.1 Calculate 95% confidence limits. What are the 95% confidence limits of a sample with a mean = 24.7, standard deviation = 6.8 and number of data values = 16? (Express your answer to three significant figures.)

77.2 Use the Poisson distribution. In a sample of 15 snails, a researcher finds the following number of parasite larvae per snail: 0, 0, 0, 0, 1, 1, 1, 3, 4, 5, 5, 7, 7, 9, 9. Using the rule of thumb on p. 605, decide whether the parasites are 'clumped' or 'repulsed' in distribution on their host. What might this mean in biological terms?

77.3 Practise using a t-test. A biology student examines the effect of adding a plant hormone to pea plants. She dissolves an appropriate amount of the compound in ethanol and applies 25 μL of this to the uppermost stipules of the treated plants. With the controls, she applies the same amount of pure ethanol. After three days, she measures the distance between the 2nd and 3rd internodes of the plants and obtains the results shown at the top of the next column. Carry out a t-test on the data and draw appropriate conclusions.

Internode distance in cm

Control	7.5	8.1	7.6	6.2	7.5	7.8	8.9
Treatment	5.6	7.5	8.2	6.7	3.5	6.5	5.9

77.4 Interpret the output from Excel linear regression analysis. The output in the two tables below represents a regression analysis for an experiment measuring the uptake of an amino acid by a cell suspension (in pmol cell^{-1}) against time (in minutes). These were obtained as output using the 'Regression' tool within the *Data > Data Analysis* menu in *Excel*. Note that 'R' here is the same as r , Pearson's product moment correlation coefficient (p. 611). Based on this output, what is the form and strength of the underlying linear relationship? (Express the coefficients to three significant figures.)

Summary output from Microsoft *Excel* spreadsheet linear regression analysis

Regression statistics	
Multiple R	0.985335951
R square	0.970886937
Adjusted R square	0.963608672
Standard error	2.133876419
Observations	6

ANOVA					
	d.f.	SS	MS	F	Significance F
Regression	1	607.4062857	607.4063	133.3954	0.000320975
Residual	4	18.21371429	4.553429		
Total	5	625.62			

	Coefficients	Standard error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	1.171428571	1.544386367	0.758507	0.490383	-3.11648428	5.459341423	-3.11648428	5.459341423
X variable 1	2.945714286	0.255047014	11.54969	0.000321	2.237588784	3.653839787	2.237588784	3.653839787

Answers to these study exercises are available at go.pearson.com/uk/he/resources

Appendix 1

Skills relevant to the biomolecular sciences and where they are covered in this textbook. The UK Subject Benchmark Statement for Biosciences (QAA, 2019a) recognises that certain skills are generic and hence common to all biosciences degrees, while others are subject-specific, where the exact nature of the skill depends on the degree topic (e.g. biochemistry, vs physiology vs genetics). The table below maps the QAA listing to relevant chapters in this book. It should be noted that there is also a UK Subject Benchmark Statement for Biomedical Sciences (QAA, 2019b), which may be relevant for certain courses, especially those with an accredited qualification.

Skill category		Examples Biomolecular sciences graduates should be able to:	Relevant chapters
A. Generic biosciences skills	Intellectual skills	<ul style="list-style-type: none"> Analyse, synthesise and summarise information critically from a variety of sources Consider issues from a number of perspectives and values and arrive at a considered critical judgement stating assumptions and limitations Construct grammatically correct documents in an appropriate academic style and format, using and referencing relevant ideas and evidence Understand the importance of academic and research integrity 	3–5, 11, 13, 15, 30
	Analytical and data interpretation skills	<ul style="list-style-type: none"> Receive and respond to a variety of sources of information: textual, numerical, verbal, graphical Understand and manipulate numerical data Solve problems by a variety of methods Determine the validity and rigour of statistical outcomes 	71–77
	Communication, presentation and information technology skills	<ul style="list-style-type: none"> Communicate about their subject appropriately to a variety of audiences, including the general public, using a range of formats and approaches and employing appropriate scientific language Cite and reference work in an appropriate manner, ensuring academic integrity and the avoidance of plagiarism whether intentional or not Use the Internet and other electronic sources critically as a means of communication and a source of information 	4, 5, 10–16
	Interpersonal and teamwork skills	<ul style="list-style-type: none"> Identify individual and collective goals and responsibilities and perform in a manner appropriate to these roles, in particular those being developed through practical, laboratory and/or field studies Recognise and respect the views and opinions of other team members Use negotiating skills Evaluate their own performance as an individual and a team member Evaluate the performance of others Develop an appreciation of the interdisciplinary nature of science and of the validity of different points of view 	6, 7, 19
	Personal and professional development skills	<ul style="list-style-type: none"> Develop the skills necessary for independent lifelong learning (for example, working independently, time management, organisational, enterprise and knowledge transfer skills) Identify and work towards targets for personal, academic, professional and career development Develop an adaptable, flexible and effective approach to study and work Build on knowledge and understanding of the role and impact of intellectual property (ip) within a research environment 	1–4, 8, 17–18, 30

Skill category	Examples Biomolecular sciences graduates should be able to:	Relevant chapters
B. Subject-specific biosciences skills	Intellectual skills <ul style="list-style-type: none"> ● Recognise and apply subject-specific theories, paradigms, concepts or principles (for example the relationship between genes and proteins, or the nature of essential nutrients in microbes, cells, plants and animals) ● Analyse, synthesise and summarise information critically, including published research or reports ● Obtain and integrate several lines of subject-specific evidence to formulate and test hypotheses ● Apply subject knowledge and understanding to address familiar and unfamiliar problems ● Recognise the moral and ethical issues of investigations and appreciate the need for ethical standards and professional codes of conduct 	5, 6, 9–16, 29–32
	Practical skills <ul style="list-style-type: none"> ● Demonstrate competence and progressive development in the basic and core experimental skills appropriate to the course of study ● Design, plan, conduct and report on investigations, which may involve primary or secondary data (for example from a survey database) ● Explain a range of appropriate and relevant experimental techniques and how they are used, and be able to perform some of them ● Devise and evaluate suitable experimental methods for the investigation of relevant areas of biochemistry and molecular biology ● Obtain, record, collate and analyse data using appropriate techniques in the field and/or laboratory, working individually or in a group, as is most appropriate for the subject under study ● Undertake field and/or laboratory investigations of living systems in a responsible, safe and ethical manner. ● Comply with health and safety policies, understand good laboratory practice,³ risk assessment, and control of substances hazardous to health assessments ● Recognise and explain the importance of quality control and quality assurance ● Recognise and explain the need for procedures for obtaining informed consent and appreciate the underlying ethical issues, including respect for the rights of access, for example, in field work or in order to map the genes of a community, family or group of plants or animals, including humans ● Demonstrate an understanding of the ethical and other issues relating to animal welfare ● Explain and justify the impact of investigations on the environment, on the organisms or subjects under investigation, and on other stakeholders 	12, 16, 19–70
	Analytical and data interpretation skills <ul style="list-style-type: none"> ● Use and interpret a variety of sources of information: textual, numerical, verbal, graphical ● Carry out sample selection; record and analyse data in the field and/or the laboratory; ensure validity, accuracy, calibration, precision, replicability and highlight uncertainty and possible bias during collection ● Mine, manipulate and interpret data from small molecule and/or macromolecular databases ● Prepare, process, interpret and present data, using appropriate qualitative and quantitative techniques, statistical courses, spreadsheets and courses for presenting data visually ● Solve problems by the most appropriate method 	6, 27, 29, 70–77

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