Chapter 1

Basic principles

1.1 BIOCHEMICAL STUDIES

1.1.1 The aims of biochemical investigations

Biochemistry is concerned with the study of the chemical processes that occur in living organisms, with the ultimate aim of understanding cell function in molecular terms. Biochemists therefore undertake studies of topics such as:

- the structural, kinetic and thermodynamic characteristics of the molecules found in the whole range of living organisms;
- the function of these molecules and the mechanisms by which they recognise and interact with each other to produce ordered anabolic, catabolic, signalling, immunological and other pathways that characterise living processes;
- the pathways that operate for the synthesis and degradation of these molecules and the mechanisms responsible for errors in the pathways;
- the energetics of biological processes, including transport across cell membranes, the generation of cellular energy, energy conversion and exchange of energy with the surrounding environment;
- the storage, replication, expression, repair, recombination and control of genetic information and the development of cell specificity.

Pioneering biochemical investigations were carried out mainly on simple prokaryotic and eukaryotic organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Neurospora crassa* and *Chlorella pyrenoidosa*. As knowledge of the nature of cellular components and control mechanisms was gained from these studies and shown to have many similarities with comparable data being gained from multicellular organisms, so the whole spectrum of biologically diverse organisms was opened up as model systems for detailed biochemical studies. Biochemists have traditionally used *in vitro* model systems rather than whole cells or organisms because of their inherently greater biochemical simplicity for experimental study and interpretation of results. Nevertheless, *in vitro* studies have the potential danger that the disruption of cells or tissues may lead to artefacts that bear little resemblance to the *in vivo* situation.

In recent years, and particularly in the past decade, the growth of biochemical knowledge relating to cellular function has grown exponentially. This has come about largely through the development of techniques for the rapid sequencing of DNA fragments released by the action of restriction enzymes, of gene cloning and site-directed mutagenesis (Chapters 5 and 6) coupled with advances in protein sequencing by mass spectrometry (Chapter 9). These developments have given rise to numerous new disciplines such as genomics (the study of cell genomes), proteomics (the study of the whole protein complement of a cell) and molecular biology, all of which fall within the broad discipline of biochemistry.

1.1.2 The design of biochemical investigations

Advances in biochemistry, as in all the sciences, are based on the careful design, execution and data analysis of experiments designed to address specific questions or hypotheses. Such experimental design involves a discrete number of compulsory stages:

- the identification of the subject for experimental investigation;
- the critical evaluation of the current state of knowledge (the 'literature') of the chosen subject area, noting the strengths and weaknesses of the methodologies previously applied and the new hypotheses that emerged from the studies;
- the formulation of the question or hypothesis to be addressed by the planned experiment;
- the careful selection of the biological system (species, *in vivo* or *in vitro*) to be used for the study;
- the identification of the variable that is to be studied; the consideration of the other variables that will need to be controlled so that the selected variable is the only factor that will determine the experimental outcome;
- the design of the experiment, including the statistical analysis of the results, careful evaluation of the materials and apparatus to be used and the consequential potential safety aspects of the study;
- the execution of the experiment, including appropriate calibrations and controls, with a carefully written record of the outcomes;
- the replication of the experiment as necessary for the unambiguous analysis of the outcomes;
- the analyses of the outcomes, including the use of appropriate statistical tests;
- the formulation of the main conclusions that can be drawn from the results;
- the formulation of new hypotheses and of future experiments that emerge from the study.

Biochemical experiments usually have much experimental detail in common. For example, the control and measurement of pH, temperature and oxygen tension are essential considerations for many studies. They also involve common manipulations, notably the preparation of solutions of known concentration and the dispensation of small volumes of reagents. The aim of this chapter is to address many of these common issues of experimental design and data analysis.

Table 1.1 SI units – basic and derived units

Quantity	SI unit	Symbol (basic SI unit)	Definition of SI unit	Equivalent in SI units
Basic units				
Length	metre	m		
Mass	kilogram	kg		
Time	second	s		
Electric current	ampere	A		
Temperature	kelvin	K		
Luminous intensity	candela	cd		
Amount of substance	mole	mol		
Derived units				
Force	newton	N	$kg\,m\,s^{-2}$	$J\,m^{-1}$
Energy, work, heat	joule	J	$kg\,m^2\,s^{-2}$	$N\,m$
Power, radiant flux	watt	W	$kg\,m^2\,s^{-3}$	$J\,s^{-1}$
Electric charge, quantity	coulomb	C	$A\,s$	$J\,V^{-1}$
Electric potential difference	volt	V	$kg\,m^2\,s^{-3}\,A^{-1}$	$J\,C^{-1}$
Electric resistance	ohm	Ω	$kg\,m^2\,s^{-3}\,A^{-2}$	$V\,A^{-1}$
Pressure	pascal	Pa	$kg\,m^{-1}\,s^{-2}$	$N\,m^{-2}$
Frequency	hertz	Hz	s^{-1}	
Magnetic flux density	tesla	T	$kg\,s^{-2}\,A^{-1}$	$V\,s\,m^{-2}$
Other units based on SI				
Area	square metre	m^2		
Volume	cubic metre	m^3		
Density	kilogram per cubic metre	$kg\,m^{-3}$		
Concentration	mole per cubic metre	$mol\,m^{-3}$		

1.2 UNITS OF MEASUREMENTS

1.2.1 SI units

The French Système International d'Unités (the SI system) is the accepted convention for all units of measurement. Table 1.1 lists basic and derived SI units. Table 1.2 lists numerical values for some physical constants in SI units. Table 1.3 lists the commonly used prefixes associated with quantitative terms. Table 1.4 gives the interconversion of non-SI units of volume.

1.2.2 Solutions – the expression of concentration

A solution is a homogeneous mixture of one or more substances (solute(s)) in a major liquid component (solvent). The concentration of the solutes in the solution expresses the amount of each solute in a given amount (weight or volume) of the solvent. The simplest expression of concentration is in terms of

Table 1.2 SI units – conversion factors for non-SI units

Unit	Symbol	SI equivalent
Avogadro constant	L or N_A	$6.022 \times 10^{23}\ mol^{-1}$
Faraday constant	F	$9.648 \times 10^{4}\ C\ mol^{-1}$
Planck constant	h	$6.626 \times 10^{-34}\ J\ s$
Universal or molar gas constant	R	$8.314\ J\ K^{-1}\ mol^{-1}$
Molar volume of an ideal gas at s.t.p.		$22.41\ dm^3\ mol^{-1}$
Velocity of light in a vacuum	c	$2.997 \times 10^{8}\ m\ s^{-1}$
Energy		
Calorie	cal	4.184 J
Erg	erg	10^{-7} J
Electron volt	eV	1.602×10^{-19} J
Pressure		
Atmosphere	atm	101 325 Pa
Bar	bar	10^5 Pa
Millimetres of Hg	mmHg	133.322 Pa
Temperature		
Centigrade	°C	(t°C + 273.15) K
Fahrenheit	°F	(t°F − 32)5/9 + 273.15 K
Length		
Ångström	Å	10^{-10} m
Inch	in.	0.0254 m
Mass		
Pound	lb	0.4536 kg

s.t.p., standard temperature and pressure.

Table 1.3 Common unit prefixes associated with quantitative terms

Multiple	Prefix	Symbol	Multiple	Prefix	Symbol
10^{24}	yotta	Y	10^{-1}	deci	d
10^{21}	zetta	Z	10^{-2}	centi	c
10^{18}	exa	E	10^{-3}	milli	m
10^{15}	peta	P	10^{-6}	micro	μ
10^{12}	tera	T	10^{-9}	nano	n
10^{9}	giga	G	10^{-12}	pico	p
10^{6}	mega	M	10^{-15}	femto	f
10^{3}	kilo	k	10^{-18}	atto	a
10^{2}	hecto	h	10^{-21}	zepto	z
10^{1}	deca	da	10^{-24}	yocto	y

weight per unit volume (w/v). Alternatives are v/v and w/w. Such expressions may also be in the form of a percentage in which case the w/v, w/w or v/v is multiplied by 100. Thus a 1% (w/v) sodium chloride solution contains 1 g NaCl in 100 cm^3 water. Less commonly, solutions may be expressed as parts per million (p.p.m.) or parts per billion (p.p.b.) of total solution. Such units can mean grams per million (or billion) grams or cm^3 in a million (or billion) grams or cm^3. Thus

Table 1.4 Interconversion of non-SI and SI units of volume

Non-SI unit	Non-SI subunit	SI subunit	SI unit
1 litre (l)	10^3 ml	$= 1\ dm^3$	$= 10^{-3}\ m^3$
1 millilitre (ml)	1 ml	$= 1\ cm^3$	$= 10^{-6}\ m^3$
1 micolitre (μl)	10^{-3} ml	$= 1\ mm^3$	$= 10^{-9}\ m^3$
1 nanolitre (nl)	10^{-6}ml	$= 1\ nm^3$	$= 10^{-12}\ m^3$

air contains approximately 8 p.p.m. carbon monoxide, where the units would be in volume. If the p.p.m relates to a solution in water, the approximation can be made that 1 g water is equivalent to 1 cm^3. Hence 8 p.p.m. would be equivalent to 8 g in 1000 dm^3 or 8 mg in 1 dm^3 or 8 μg in 1 cm^3 or 8 ng in 1 mm^3 (see Table 1.4).

Molarity

The SI unit of the amount of any substance is the mole, defined as the amount that contains Avogadro's number (N_A) of molecules (6.022×10^{23}). It can also be defined as the amount of a substance in which the number of elementary entities is equal to Avagadro's constant. It is therefore possible to have a mole of molecules, of atoms, of ions or even of electrons. In practical terms, one mole of a substance is equal to its molecular mass expressed in grams, where the molecular mass is the sum of the atomic masses of the constituent atoms. Note that the term molecular mass is preferred to the older term molecular weight. The SI unit of concentration is expressed in terms of moles per cubic metre (mol m^{-3}) (see Table 1.1). In practice this is far too large for normal laboratory purposes and a unit based on a cubic decimetre (dm^3, 10^{-3} m) is preferred. However, some textbooks and journals, especially those of North American origin, tend to use the older unit of volume, namely the litre and its subunits (see Table 1.4) rather than cubic decimetres. In this book, volumes will be expressed in cubic decimetres or its smaller counterparts (Table 1.4). The molarity of a solution of a substance expresses the number of moles of the substance in 1 dm^3 of solution. Molarity is expressed by the symbol M, but, since this has another use in the SI system (mega = M), mol dm^{-3} is recommended instead. Nevertheless, molarity continues to be expressed as M in many textbooks and international journals as well as in conversation and will be used in this book.

It should be noted that atomic and molecular masses are both expressed in daltons (Da) or kilodaltons (kDa), where one dalton is an atomic mass unit equal to one-twelfth of the mass of one atom of the ^{12}C isotope. However, biochemists prefer to use the term relative molecular mass (M_r). This is defined as the molecular mass of a substance relative to one-twelfth of the atomic mass of the ^{12}C isotope. M_r therefore has no units. Thus the relative molecular mass of sodium chloride is 23 (Na) plus 35.5 (Cl), i.e. 58.5, so that one mole is 58.5 g. If this was dissolved in water and adjusted to a total volume of 1 dm^3, the solution would be one molar (1 M).

Table 1.5 Interconversion of mol, mmol and μmol in different volumes to give different concentrations

Molar (M)	Millimolar (mM)	Micromolar (μM)
1 mol dm^{-3}	1 mmol dm^{-3}	1 μmol dm^{-3}
1 mmol cm^{-3}	1 μmol cm^{-3}	1 nmol cm^{-3}
1 μmol mm^{-3}	1 nmol mm^{-3}	1 pmol mm^{-3}

Biological substances are most frequently found at relatively low concentrations and in *in vitro* model systems the volumes of stock solutions regularly used for experimental purposes are also small. The consequence is that experimental solutions are usually in the mmol dm^{-3}, μmol dm^{-3} and nmol dm^{-3} range rather than molar. Table 1.5 shows the interconversion of these units.

Example 1 CALCULATION OF MOLARITY

Question 1

How would you prepare 250 cm^3 of 0.1 M glucose?

Answer

The molecular formula of glucose is $C_6H_{12}O_6$ so its molecular mass is $(6 \times 12) + (12 \times 1) + (6 \times 16)$, i.e. 180 daltons. Hence, 180 g dissolved in 1 dm^3 would give a 1 M solution, so that 18 g dissolved in 1 dm^3 would give a 0.1 M solution. Hence to prepare 250 cm^3 of 0.1 M solution, 4.5 g of glucose would be dissolved in water and the total volume adjusted to 250 cm^3 in a volumetric flask.

Question 2

How would you prepare 10 cm^3 of 0.01 M glucose from a 0.1 M stock solution?

Answer

Applying the dilution formula, $M_1 V_1 = M_2 V_2$, $M_1 = 0.1$, V_1 is unknown, $M_2 = 0.01$, $V_2 = 10$ cm^3. Hence $0.1 \times V_1 = 0.01 \times 10$, so V_1 must be 1.0 cm^3. Hence 1.0 cm^3 stock solution (dispensed via an accurate automatic pipette) would be diluted to 10 cm^3 in a volumetric flask.

Question 3

What is the approximate molarity of a solution of glucose that contains 20 p.p.m.?

Answer

A 20 p.p.m. solution contains 20 g in one million grams or 20 mg in one kilogram. Assuming that the density of the solution is 1 g cm^{-3}, this is equivalent to 20 mg dm^{-3}. Hence the molarity of the solution is $20 \times 10^{-3}/180$ M, i.e. 0.11×10^{-3} M or 0.11 M.

Question 4

What is the molarity of pure water?

Answer

Water has a molecular mass of $2 + 16 = 18$ daltons. The molarity of 1 dm^3 of water (equivalent to 1000 g if the density is assumed to be 1 g cm^{-3}) is therefore equal to 1000/18, i.e. 55.6 M.

Dilution

In the preparation of experimental solutions it is common practice to prepare dilute solutions from more concentrated stock solutions. This dilution is easily achieved using the formula $M_1V_1 = M_2V_2$, where M_1 and M_2 represent the initial and final molarities and V_1 and V_2 represent the initial and final volumes. For the preparation of a given dilution, three of the variables will be known and the fourth can be calculated.

1.2.3 Concentration or activity?

Ionisation and ionic strength

A solution of sodium chloride in water does not contain molecules of NaCl but rather individual sodium (Na^+) and chloride (Cl^{-1}) ions due to the process of ionisation. Ionisation is possible in this case because sodium chloride forms a crystal lattice in which the sodium and chloride ions are held together by purely ionic attraction, i.e. there is no covalent bond formation. Sodium chloride is typical of the majority of inorganic salts, all of which ionise more or less completely in solution. The process of ionisation in such cases is therefore shown as being irreversible. Collectively these salts are said to be strong electrolytes and contrast with many other compounds, mainly organic acids and bases, which are only partially ionised in solution and are therefore said to be weak electrolytes. The process of ionisation of weak electrolytes is shown as being reversible. Ionisation of weak electrolytes, such as the carboxylic acids, is possible because, although there is a covalent bond between the oxygen and hydrogen atoms in the carboxyl group, the bond is highly polarised so that there pre-exists a partial positive charge on the hydrogen atom. Ionisation to release a proton places a negative charge on the oxygen atom that can be delocalised over the other oxygen atom in the carboxyl group. This stabilises the carboxyl anion (COO^-) relative to the carboxyl group and encourages ionisation. The relative ease with which the ionisation of weak electrolytes occurs is discussed in Section 1.3. Yet other organic compounds, for example alcohols including simple sugars such as glucose, do not ionise at all in solution and are therefore said to be non-electrolytes.

For some biochemical studies involving the use of both strong and weak electrolytes it is more important to measure the amount of individual ions present in solution than to know the concentration of the compound from which they arise. Ionic strength (μ) is a measure of the total ion charge in solution and is determined by both the concentration of all the individual ions present and their charge. Its value is calculated by use of equation 1.1:

$$\mu = \frac{1}{2}(c_1z_1^2 + c_2z_2^2 \cdots c_nz_n^2)$$

$$= \frac{1}{2}\Sigma cz^2 \qquad (1.1)$$

where Σ indicates the sum of all the terms of the following type, c_1, $c_2 \ldots c_n$ is the concentration of each individual ion in molarity, and z_1, $z_2 \ldots z_n$ is the charge on the individual ions (thus for Na^+ and K^+ $z = +1$, for Cl^- and NO_3^- $z = -1$, for Ca^{2+} $z = +2$, and for $SO_4^{2-} = -2$).

Whilst salts such as NaCl and KNO_3, which consist of monovalent ions, ionise almost completely in aqueous solution, those that consist of divalent ions, such as $MgSO_4$, ionise to a smaller extent owing to the process of ion-pairing. In this process, ions of opposite charge are attracted to each other in aqueous solution to form a tightly bound ion-pair that behaves as a single particle. Thus a 0.25 M solu-

Example 2 CALCULATION OF IONIC STRENGTHS

Question

Calculate the ionic strength of (i) 0. 1 M NaCl, (ii) 0. 1 M NaCl + 0.05 M KNO_3 + 0.01 M Na_2SO_4.

Answer

Ionic strength can be calculated using the equation $\mu = \frac{1}{2}\sum cz^2$.

(i) Calculating cz^2 for each ion:

$$Na^+ = 0.1 \times (+1)^2 = 0.1\,M$$

$$Cl^- = 0.1 \times (-1)^2 = 0.1\,M$$

Hence

$$\frac{1}{2}\sum cz^2 = 0.2/2 = 0.1\,M$$

(ii)

$$Na^+ = 0.1 \times (+1)^2 + 0.02 \times (+1)^2 = 0.12\,M$$

$$Cl^- = 0.1 \times (-1)^2 = 0.10\,M$$

$$K^+ = 0.05 \times (+1)^2 = 0.05\,M$$

$$NO_3^- = 0.05 \times (-1)^2 = 0.05\,M$$

$$SO_4^{2-} = 0.01 \times (-2)^2 = 0.04\,M$$

Hence

$$\frac{1}{2}\sum cz^2 = \frac{1}{2}(0.36) = 0.18\,M$$

Note 1: the unit of ionic strength is M.

Note 2: that for Na_2SO_4 $c = 0.02$, since there are $2Na^+$ per mole.

Note 3: that for a 1 M 1 : 1 electrolyte such as NaCl, the ionic strength is 1 M ; for a 1 M 2 : 1 electrolyte such as $MgCl_2$, the ionic strength is 3 M , for a 1 M 2 : 2 electrolyte such as $MgSO_4$, the ionic strength is 4 M and for a 3 : 1 electrolyte such as $FeCl_3$, the ionic strength is 6 M .

Note 4: As the concentration and ionic strength increase, this type of calculation becomes progressively inaccurate owing to the importance of activity coefficients.

tion of $MgSO_4$ is 65% ionised (i.e. 65% consists of individual magnesium and sulphate ions in solution), the remaining 35% existing as ion-pairs. As a consequence of ion-pairing it is more difficult to calculate the ionic strength of solutions of salts of this type.

In aqueous solution, anions and cations are surrounded by an ionic atmosphere or shell owing to the attraction by the ion of oppositely charged species, including water molecules in which the O—H bond is polarised to give a δ^+ on each of the hydrogen atoms and δ^- on the oxygen atom. This ionic atmosphere has a net charge that is opposite to, but smaller than, that of the ion it surrounds. Its presence results in a reduction of the effective charge of the central ion and hence its attraction for oppositely charged ions. This effect is enhanced by an increase in the ionic strength of the solution. This is the basis of the salting out of proteins (Section 8.3.4).

Activities and activity coefficients

Ionic strength influences the effective concentration of a compound that can ionise in solution such that the effective concentration, referred to as the *activity* (*A*), is related to the nominal concentration by a factor known as *the activity coefficient* (γ) as shown in equation 1.2:

$$A_X = [X]\,\gamma_x \tag{1.2}$$

where A_X is the activity of species X, [X] is the concentration of X, and γ_x is the activity coefficient of X. An activity coefficient is a measure of the deviation of the behaviour of a species from the expected. As the ionic strength increases, the activity coefficient decreases, reducing the activity relative to the concentration. Thus the activity coefficient for Na^+ is 0.964 at 0.001 M and 0.79 at 0.1 M. The reverse of this, namely that, as the ionic strength decreases, the activity coefficient approaches unity, is important, since it means that under these circumstances the activity and concentration of the ion converge. The implications of this will be discussed later in the context of pH (Section 1.3.2). If the ionisable species gives rise to multiply charged ions, the activity coefficient decreases, irrespective of the sign, + or −, of the ions. Thus the activity coefficient for Mg^{2+} and Fe^{3+}, each at 0.001 M, is 0.872 and 0.738, respectively, at 25 °C.

Practical biochemical studies quite commonly include the use of reactants that are subject to discrepancies between concentration and activity. The design of such experiments has to include an assessment of the importance of this effect. Clearly, the impact will be greatest in those cases where the effect of a particular ion on a process or response is being studied. It is also important to realise that electrodes, such as the pH electrode and ion-selective electrodes commonly used in biochemical work, respond to the activities rather than concentration of the ion being measured. In the majority of other types of study it is generally acceptable to assume that activity and concentration are interchangeable, bearing in mind that in most biochemical studies the concentrations of reagents are generally low. When needed, the values of activity coefficients of organic and inorganic ions can be found in tables of physical constants.

1.3 WEAK ELECTROLYTES

1.3.1 The biochemical importance of weak electrolytes

Many molecules of biochemical importance are weak electrolytes in that they are acids or bases that are only partially ionised in aqueous solution. Examples include the amino acids, peptides, proteins, nucleosides, nucleotides and nucleic acids. The biochemical function of many of these molecules is dependent upon their precise state of ionisation at the prevailing cellular or extracellular pH. The catalytic sites of enzymes, for example, contain functional carboxyl and amino groups, from the side-chains of constituent amino acids in the protein chain, that need to be in a specific ionised state to enable the catalytic function of the enzyme to be realised. Before the ionisation of these compounds is discussed in detail, it is necessary to appreciate the importance of the ionisation of water.

1.3.2 Ionisation of weak acids and bases

Ionisation of water

One of the most important weak electrolytes is water, since it ionises to a small extent to give hydrogen ions and hydroxyl ions. In fact there is no such species as a free hydrogen ion in aqueous solution, as it reacts with water to give a hydronium ion (H_3O^+):

$$H_2O \rightleftharpoons H^+ + HO^-$$

$$H^+ + H_2O \rightleftharpoons H_3O^+$$

Even though free hydrogen ions do not exist, it is conventional to refer to them rather than hydronium ions. The equilibrium constant for the ionisation of water has a value of 1.8×10^{-16} at 24 °C. Since the ionic strength of water is very low, the activity coefficients for the hydrogen ions and hydroxyl ions will both effectively be unity so that the activity of each of these two ions is equal to their concentration. As calculated previously, the molarity of pure water is 55.6. This can be incorporated into a new constant, K_w. Thus, effectively, the activity of water is set at unity. It follows that:

$$1.8 \times 10^{-16} \times 55.6 = [H^+][HO^-] = 1.0 \times 10^{-14} = K_w \quad (1.3)$$

K_w is known as the autoprotolysis constant of water and does not include an expression for the concentration of water. Its numerical value of exactly 10^{-14} relates specifically to 24 °C. At 0 °C K_w has a value of 1.14×10^{-15} and at 100 °C a value of 5.45×10^{-13}. The stoichiometry in equation 1.3 shows that hydrogen ions and hydroxyl ions are produced in a 1 : 1 ratio; hence both of them must be present at a concentration of 1.0×10^{-7} M. Since the Sörensen definition of pH is that it is equal to the negative logarithm of the hydrogen ion activity, it follows that the pH of pure water is 7.0. This is the definition of neutrality.

This theoretical background to the pH of water is well known, but what is not so well appreciated is the influence on the pH of water of adding a salt such as NaCl.

Salt is a strong electrolyte and is virtually completely ionised in dilute aqueous solution, but it does not result in the production of either hydrogen ions or hydroxyl ions. It might therefore be expected that it would not affect the pH of water. This assumption, however, ignores the concomitant change in the ionic strength of the solution. For example, if NaCl is added to a concentration of 0.1 M, the activity coefficients of the hydrogen ions and hydroxyl ions are lowered to 0.83 and 0.76, respectively. Hence the following position now prevails:

$$K_w = 1.0 \times 10^{-14} = [H^+]0.83 \times [HO^-]0.76$$

As before, the hydrogen ions and hydroxyl ions are produced in equal amounts so that it can be calculated from the above that each is equal to 1.26×10^{-7} M. The activity of the hydrogen ions in solution is therefore equal to $1.26 \times 10^{-7} \times 0.83 = 1.05 \times 10^{-7}$ M. Hence the pH of the 0.1 M NaCl is 6.90. Whilst this may seem a very small change from that of the original pure water, this is misleading owing to the logarithmic nature of pH as it actually represents a 26% increase in the hydrogen ion concentration and 20% in hydrogen ion activity relative to that of pure water. Once again, this highlights the importance of considering activities rather than concentrations when the ionic strength of an experimental solution could influence the interpretation of experimental data.

Ionisation of carboxylic acids and amines

As previously stressed, many biochemically important compounds contain a carboxyl group (-COOH) or a primary (RNH_2), secondary (R_2NH) or tertiary (R_3N) amine that can donate or accept a hydrogen ion on ionisation. The tendency of a weak acid, generically represented as HA, to ionise is expressed by the equilibrium reaction:

$$\underset{\text{weak acid}}{HA} \rightleftharpoons H^+ + \underset{\substack{\text{conjugate}\\ \text{base (anion)}}}{A^-}$$

This reversible reaction can be represented by an equilibrium constant, K_a, known as the acid dissociation constant (equation 1.4). Numerically, it is very small.

$$K_a = \frac{[H^+][A^-]}{[HA]} \tag{1.4}$$

Note that the ionisation of a weak acid results in the release of a hydrogen ion and the conjugate base of the acid, both of which are ionic in nature.

Similarly, amino groups (primary, secondary and tertiary) as weak bases can exist in ionised and unionised forms and the concomitant ionisation process represented by an equilibrium constant, K_b (equation 1.5):

$$\underset{\substack{\text{weak base}\\ \text{(primary amine)}}}{RNH_2} + H_2O \rightleftharpoons \underset{\substack{\text{conjugate acid}\\ \text{(substituted}\\ \text{ammonium ion)}}}{RNH_3^+} + HO^-$$

$$K_b = \frac{[RNH_3^+][HO^-]}{[RNH_2][H_2O]} \tag{1.5}$$

In this case, the non-ionised form of the base abstracts a hydrogen ion from water to produce the conjugate acid, which is ionised. If this equation is viewed from the reverse direction it is of a format similar to that of equation 1.4. Equally, equation 1.4 viewed in reverse is similar in format to equation 1.5.

From our previous consideration of concentration, activity and activity coefficients, it is evident that equations 1.2 and 1.3 are incorrect in that they lack an activity coefficient term for each of the reaction species. Thus the correct form of equation 1.4 is:

$$K_a = \frac{[H^+]_{\gamma_{H^+}}[A^-]_{\gamma_{A^-}}}{[HA]_{\gamma_{HA}}} \tag{1.6}$$

where γ_H^+, γ_A^- and γ_{HA} are the activity coefficients for the three species.

The practical implications of including expressions for the activity coefficients will be discussed later but, in general, if the difference between concentration and activity of a species under investigation is such as to compromise the quantitative outcome to the study, then activity coefficients must be taken into account.

A specific and simple example of the ionisation of a weak acid is that of acetic (ethanoic) acid, CH_3COOH:

$$\underset{\text{acetic acid}}{CH_3COOH} \rightleftharpoons \underset{\text{acetate anion}}{CH_3COO^-} + H^+$$

Acetic acid and its conjugate base, the acetate anion, are known as a conjugate acid–base pair. The acid dissociation constant can be written as:

$$K_a = \frac{[CH_3COO^-]_{\gamma_{Ac}}[H^+]_{\gamma_{H^+}}}{[CH_3COOH]_{\gamma_{HAc}}} = \frac{[\text{conjugate base}]_{\gamma_{Ac}}[H^+]_{\gamma_{H^+}}}{[\text{weak acid}]_{\gamma_{HAc}}}$$

where γ_{HAc} and γ_{Ac} are the activity coefficients for acetic acid and the acetate anion respectively.

K_a has a value of 1.75×10^{-5}. Hence its negative logarithm, pK_a (i.e. $-\log K_a$), is equal to 4.75. It can be seen from equation 1.4 that pK_a is numerically equal to the pH at which 50% of the acid is protonated (unionised) and 50% is deprotonated (ionised).

It is possible to write an expression for the K_b of the acetate anion as a conjugate base:

$$CH_3COO_3^- + H_2O \rightleftharpoons CH_3COOH + HO^-$$

$$K_b = \frac{[CH_3COOH]_{\gamma_{HAc}}[HO^-]_{\gamma_{Ho^{2-}}}}{[CH_3COO^-]_{\gamma_{HAc}}}$$

K_b has a value of 1.77×10^{-10}, hence $pK_b = 9.25$.

Multiplying these two expressions together results in the important relationship:

$$K_a \times K_b = K_w = 1.0 \times 10^{-14} \text{ at } 24\,^{\circ}\text{C}$$

hence

$$pK_a + pK_b = pK_w = 14 \tag{1.7}$$

This relationship holds for all acid–base pairs and enables one pK_a value to be calculated from knowledge of the other.

Many biologically important molecules are amphoteric, i.e. they have both acidic and basic groups and therefore in aqueous solution can accept or donate protons. The amino acids are a case in point and this aspect of their properties is discussed in Section 8.1.

In the case of the ionisation of weak bases, the most common convention is to quote the K_a or the pK_a of the conjugate acid rather than the K_b or pK_b of the weak base itself. Examples of the pK_a values of some weak acids and bases are given in Table 1.6. Remember that the smaller the numerical value of pK_a the stronger the acid (more ionised) and the weaker its conjugate base. Weak acids will be predominantly unionised at low pH values and ionised at high values. In contrast, weak bases will be predominantly ionised at low pH values and unionised at high values. This sensitivity to pH of the state of ionisation of weak electrolytes is important both physiologically and in *in vitro* studies employing such analytical techniques as electrophoresis and ion-exchange chromatography.

Ionisation of polyprotic weak acids and bases

Polyprotic weak acids and bases are capable of donating or accepting more than one hydrogen ion. Each ionisation stage can be represented by a K_a value using the convention that K_a^1 refers to the acid with the most ionisable hydrogen atoms and K_a^n the acid with the least number of ionisable hydrogen atoms. One of the most important biochemical examples is phosphoric acid, H_3PO_4:

$$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \qquad pK_a^1 \quad 1.96$$

$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \qquad pK_a^2 \quad 6.70$$

$$HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-} \qquad pK_a^3 \quad 12.30$$

Example 3 CALCULATION OF pH AND THE EXTENT OF IONISATION OF A WEAK ELECTROLYTE

Question

Calculate the pH of a 0.01 M solution of acetic acid and its fractional ionisation given that its K_a is 1.75×10^{-5}.

Answer

To calculate the pH we can write:

$$K_a = \frac{[\text{acetate}^-][\text{H}^+]}{[\text{acetic acid}]} = 1.75 \times 10^{-5}$$

Since acetate and hydrogen ions are produced in equal quantities, if $x =$ the concentration of each then the concentration of unionised acetic acid remaining will be $0.01 - x$. Hence:

$$1.75 \times 10^{-5} = \frac{(x)(x)}{0.01 - x}$$

$$1.75 \times 10^{-7} - 1.75 \times 10^{-5}x = x^2$$

This can now be solved either by use of the quadratic formula or, more easily, by neglecting the x term since it is so small. Adopting the latter alternative gives:

$$x^2 = 1.75 \times 10^{-7}$$

hence

$$x = 4.18 \times 10^{-4}\text{M}$$

hence

$$\text{pH} = 3.38$$

Note that this solution has ignored the activity coefficients of the acetate and hydrogen ions. They are 0.90 and 0.91 respectively at 0.01 M and 25 °C. Inserting these values into the above expression and assuming that the activity coefficient of acetic acid is unity gives:

$$1.75 \times 10^{-5} = \frac{(x)0.90(x)0.91}{0.01 - x}$$

Solving this equation for x gives a value of 4.61×10^{-4}M, and hence a pH of 3.33. This illustrates the relatively small influence of activity coefficients in this case.

The fractional ionisation (α) of the acetic acid is defined as the fraction of the acetic acid that is in the form of acetate and is therefore given by the equation:

$$\alpha = \frac{[\text{acetate}]}{[\text{acetate}] + [\text{acetic acid}]}$$

$$= \frac{4.18 \times 10^{-4}}{4.18 \times 10^{-4} + 0.01 - 4.18 \times 10^{-4}}$$

$$= \frac{4.18 \times 10^{-4}}{0.01}$$

$$= 4.18 \times 10^{-2} \quad \text{or } 4.18\%$$

Thus the majority of the acetic acid is present as the unionised form. If the pH is increased above 3.33 the proportion of acetate present will increase in accordance with the Henderson–Hasselbalch equation.

1.4 BUFFER SOLUTIONS – THEIR NATURE AND PREPARATION

1.4.1 Titration curves

If a solution of a weak acid, such as acetic acid, is titrated with a strong base, such as sodium hydroxide, and the change in pH monitored continuously by use of a calibrated pH electrode and meter, it will be observed that the initial pH of around 3 (see Example 3) will gradually increase then begin to plateau as half-neutralisation is reached. As the titration continues, this plateau of small change in pH eventually ceases and the pH increases again more rapidly until the acid is fully neutralised. The same shape of titration curve will be observed irrespective of the weak acid chosen, the only differences being the initial pH and the prevailing pH in the region of half-neutralisation. If a diprotic acid, such as succinic acid, is titrated, two plateau regions will be observed and in the case of a triprotic acid, such as phosphoric acid, three plateau regions will be evident. In all cases the pH at the mid-point of the plateau region will be equal to the pK_a of the acid and the extent of the plateau approximately $pK_a \pm 1$ pH unit. This plateau region gives a clue as to the nature of a buffer solution; that is, one that resists a change in pH on the addition of acid or alkali. At half-neutralisation the titration solution contains equal amounts of the conjugate base of the acid and unionised acid. This is the characteristic of all buffer solutions. The conjugate base neutralises added acid whilst the unionised acid neutralises added base.

1.4.2 Henderson–Hasselbalch equation

The Henderson–Hasselbalch equation is of central importance in the preparation of buffer solutions. It can be expressed in a variety of forms. For a buffer based on a weak acid:

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\text{conjugate base}]}{[\text{weak acid}]} \tag{1.8a}$$

or

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\text{ionised form}]}{[\text{unionised form}]} \tag{1.8b}$$

For a buffer based on the conjugate acid of a weak base:

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\text{weak acid}]}{[\text{conjugate base}]} \tag{1.9a}$$

or

$$\mathrm{pH} = \mathrm{p}K_a + \log \frac{[\text{unionised form}]}{[\text{ionised form}]} \tag{1.8b}$$

For total correctness, all four forms should contain expressions for the activity coefficients of each species, but these have been omitted for clarity.

1.4.3 Buffer capacity

It can be seen from the Henderson–Hasselbalch equation that when the concentration (or more strictly the activity) of the weak acid and base is equal, their ratio is 1 and their logarithm zero so that $\mathrm{pH} = \mathrm{p}K_a$. This rationalises the shape of titration curves with their plateau at the mid-point of neutralisation. The ability of

Table 1.6 **pK_a values of some acids and bases that are commonly used as buffer solutions**

Acid or base	pK_a
Acetic acid	4.75
Barbituric acid	3.98
Carbonic acid	6.10, 10.22
Citric acid	3.10, 4.76, 5.40
Glycylglycine	3.06, 8.13
Hepes[a]	7.50
Phosphoric acid	1.96, 6.70, 12.30
Phthalic acid	2.90, 5.51
Pipes[a]	6.80
Succinic acid	4.18, 5.56
Tartaric acid	2.96, 4.16
Tris[a]	8.14

[a] See list of abbreviations at the front of the book.

a buffer solution to resist a change in pH on the addition of strong acid or alkali is expressed by its buffer capacity (β). This is defined as the amount (moles) of acid or base required to change the pH by one unit, i.e.:

$$\beta = \frac{db}{dpH} = \frac{-da}{dpH} \tag{1.10}$$

where db and da are the amount of base and acid respectively, and dpH is the resulting change in pH. In practice, β is largest within the pH range p$K_a \pm 1$ confirming the earlier observation about the size of the plateau in the titration curve. Table 1.6 lists some weak acids and bases commonly used in the preparation of buffer solutions.

1.4.4 Preparation of buffer solutions

Buffer solutions may be prepared either by adding a strong base, such as 0.5 M NaOH, to the calculated quantity of the weak acid, based on a Henderson–Hasselbalch calculation, or by mixing the calculated quantities of the weak acid and its sodium salt. In both cases the resulting mixture is adjusted to a volume just short of the required volume, checked for the correct pH using a calibrated pH electrode and pH meter and finally adjusted to the correct total volume in a volumetric flask. Methods based on the addition of the calculated quantities of weak acid and its conjugate weak base should, in principle, automatically give rise to the required pH but in practice this may not be the case for a number of reasons including, first, that most commonly the calculations will have been based on molarities rather than activities and, secondly, because the temperature and hence the pK_a may not be correct. As previously emphasised, pH is sensitive to the ionic strength of the solution so appropriate precautions must be taken when adding other reagents to a stock buffer solution. However, simple dilution of a stock buffer solution should have little impact on its pH. It will, however, decrease the buffer capacity.

Selection of a buffer

When selecting a buffer for a particular experimental study, several factors should be taken into account:

- select the one with a pK_a as near as possible to the required experimental pH and within the range $pK_a \pm 1$, as outside this range there will be too little weak acid or weak base present to maintain an effective buffer capacity;
- select an appropriate concentration of buffer to have adequate buffer capacity for the particular experiment. Most commonly buffers are used in the range 0.05–0.5 M;
- ensure that the selected buffer does not form insoluble complexes with any anions or cations essential to the reaction being studied (phosphate buffers tend to precipitate polyvalent cations, for example, and may be a metabolite or inhibitor of the reaction);
- ensure that the proposed buffer has other desirable properties such as being non-toxic, able to penetrate membranes, and does not absorb in the visible or ultraviolet region.

Example 4 PREPARATION OF A PHOSPHATE BUFFER

Question

How would you prepare 1 dm^3 of 0.1 M phosphate buffer, pH 7.1, given that pK_a^2 for phosphoric acid is 6.8 and that the atomic masses for Na, P and O are 23, 31 and 16 daltons respectively?

Answer

The buffer will be based on the ionisation:

$$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+ \qquad pK_a^2 = 6.8$$

and will therefore involve the use of solid sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4).

Applying the appropriate Henderson–Hasselbalch equation (equation 1.8) gives:

$$7.1 = 6.8 + \log\frac{[HPO_4^{2-}]}{[H_2PO_4^-]}$$

$$0.3 = \log\frac{[HPO_4^{2-}]}{[H_2PO_4^-]^2}$$

$$2.0 = \frac{[HPO_4^{2-}]}{[H_2PO_4^-]^2}$$

Since the total concentration of the two species needs to be 0.1 M it follows that $[H_2PO_4^{2-}]$ must be 0.067 M and $[H_2PO_4^-]$ 0.033 M. Their molecular masses are 142 and 120 daltons, respectively; hence the weight of each required is $0.067 \times 142 = 9.51$ g (Na_2HPO_4) and $0.033 \times 120 = 3.96$ g (NaH_2PO_4). These weights would be dissolved in approximately 800 cm^3 of pure water, the pH measured and adjusted as necessary, and the volume finally made up to 1 dm^3.

1.5 pH AND OXYGEN ELECTRODES

1.5.1 Reference electrodes

Half-cells and galvanic cells

One of the most common needs in biochemical experiments is the requirement to measure and control the pH (i.e. the hydrogen ion activity) of a reaction mixture. A close second, in terms of routine need, is the measurement of the oxygen tension of a solution. These needs are best met by the use of special electrodes that respond specifically to the particular ion or molecule (the analyte). These electrodes work on the basis that the analyte in question can accept an electron from, or donate one to, an electrode, which is most commonly made of platinum. This transfer of electrons is the basis of oxidation and reduction reactions: acceptance of an electron by the analyte resulting in its reduction; the donation of an electron by the analyte resulting in its oxidation. In general terms an oxidation–reduction reaction such as this can be represented as follows, where X is a chemical species capable of being reduced, Y a species capable of being oxidised, e^- is an electron and a, n and b are small integers:

$$\underset{\text{oxidised analyte}}{a\text{X}} + n\text{e}^- \rightleftharpoons \underset{\text{reduced analyte}}{b\text{Y}}$$

The transfer of electrons to or from the electrode establishes a so-called indicator (or sensing) half-cell and a potential (E) at the electrode. This potential can be quantified by coupling the half-cell to a reference half-cell, which generates a constant and known potential so that the experimentally measured potential, which is the net sum of the potentials generated by the two half-cells, can be correlated to that produced by the indicator half-cell. The two half-cells must be linked via a salt bridge, which allows electrons to flow between the two, thus maintaining electrical neutrality. Two half-cells linked in this manner constitute a galvanic cell.

The experimental potential (E) generated by any half-cell is given by the Nernst equation:

$$\begin{aligned} E &= E^0 - 2.303\,(RT/nF)\log\,(A_\text{Y}/A_\text{X}) \\ &= E^0\,(-0.0592/n)\log\,(A_\text{Y}/A_\text{X}) \text{ volts at } 25\,^\circ\text{C} \end{aligned} \qquad (1.11)$$

where E^0 is the standard reduction potential (also called a standard potential or a standard redox potential), A_Y and A_X are the activities of the reduced and oxidised species respectively, F is the Faraday (electrical charge of one mole of electrons) ($9.648 \times 10\,\text{J V}^{-1}\,\text{mol}^{-1}$), R is the molar gas constant ($8.314\,\text{J mol}^{-1}\,\text{K}^{-1}$), n is the number of moles of electrons per mole of reactant transferred in the half-cell, T is the absolute temperature, $(2.303RT)/F$ is the Nernst factor that at 25 °C is equal to 0.059159 V.

When the activities of the oxidised and reduced species are equal, their ratio will be one and their logarithm zero so that $E = E^0$. It is important to note that it is

the ratio of the two activities rather than their absolute values that determines the value of the experimental potential *E*. The Nernst equation therefore defines the potential of a half-cell whose reactive species are not present at unit activity.

The calomel electrode and the silver/silver chloride electrode are the two most commonly used reference electrodes to measure the potential of an indicator half-cell.

Saturated calomel electrode

This consists of two concentric tubes, the smaller central one containing a platinum electrode in contact with a paste of mercury, mercurous chloride and potassium chloride. At the base of this inner tube is a small opening to the outer tube, which contains a saturated solution of potassium chloride as the salt bridge to link the half-cell to the indicator half-cell via a porous plug (Fig. 1.1a). The half-cell can be represented as follows;

$Hg|Hg_2Cl_2|KCl||$ test solution

where the double line represents the salt bridge linking the half-cell to the indicator half-cell.

The calomel electrode generates a potential according to the following equation:

$$\underset{\substack{\text{mercurous chloride}\\ \text{(calomel)}}}{\frac{1}{2}Hg_2Cl_2} + e^- \rightleftharpoons \underset{\text{mercury}}{Hg} + Cl^-$$

The standard reduction potential (E^0) of the half-cell is +0.241 V.

Silver/silver chloride electrode

This consists of a deposit of silver chloride on the surface of metallic silver inserted in a saturated solution of silver chloride and potassium chloride contained in a tube the end of which is open to the test solution via a porous plug covered with solid potassium chloride. The half-cell generates a potential according to the following reaction;

$$AgCl + e^- \rightleftharpoons Ag + Cl^-$$

The standard reduction potential (E^0) of the half-cell is +0.197 V.

The use of saturated potassium chloride in both these two reference electrodes ensures that the concentrations of their reactive species remain constant.

Standard hydrogen electrode

Although the calomel and silver/silver chloride reference electrodes are in common use, their standard reduction potentials have in turn to be measured against an international standard. This is the standard hydrogen electrode. It consists of a platinum electrode in a solution of hydrochloric acid, the activity of which is unity, through which hydrogen gas at one atmosphere pressure

Table 1.7 Standard redox potentials of biochemical interest (at 25 °C)

$E^{0\prime}$(V)	Half-reaction
−0.42	$2H^+ + 2e^- \rightleftharpoons H_2$
−0.32	$NAD^+ + H^+ + 2e^- \rightleftharpoons NADH$
−0.22	$FAD + 2H^+ + 2e^- \rightleftharpoons FADH_2$
−0.20	Acetaldehyde $+ 2H^+ + 2e^- \rightleftharpoons$ ethanol
−0.19	Pyruvate $+ 2H^+ + 2e^- \rightleftharpoons$ lactate
−0.17	Oxaloacetate $+ 2H^+ + 2e^- \rightleftharpoons$ malate
−0.03	Fumarate $+ 2H^+ + 2e^- \rightleftharpoons$ succinate
+0.08	Cytochrome b (Fe^{3+}) $+ e^- \rightleftharpoons$ cytochrome b (Fe^{2+})
+0.25	Cytochrome c (Fe^{3+}) $+ e^- \rightleftharpoons$ cytochrome c (Fe^{2+})
+0.29	Cytochrome a (Fe^{3+}) $+ e^- \rightleftharpoons$ cytochrome a (Fe^{2-})
+0.30	$\frac{1}{2}O_2 + H_2O + 2e^- \rightleftharpoons H_2O_2$
+0.80	$O_2 + 2H^+ + 2e^- \rightleftharpoons H_2O$

(101 325 Pa) (so that its activity is also unity) is bubbled to enable the following equilibrium to be established:

$$H^+ + e^- \rightleftharpoons \frac{1}{2}H_2$$

When the activities of the hydrogen ions and hydrogen gas are unity, the electrode is arbitrarily assigned a standard reduction potential of 0.00V at 25 °C. The electrode is too cumbersome for routine laboratory use.

The use of standard reduction potentials (E^0), developed initially by chemists, presents biochemists with a difficulty, since they are based on unit activity of hydrogen ions (i.e. pH 0). The majority of biochemical reactions occurring in living cells take place around neutrality (i.e. pH 7). Biochemists have therefore introduced an alternative scale of standard reduction potentials based on pH 7. These are referred to as $E^{0\prime}$ (spoken as E nought dash). These values are less positive than their E^0 counterparts. Table 1.7 gives some examples of standard redox potentials of biochemical interest. Positive $E^{0\prime}$ values represent a more oxidising system and negative values a more reducing system than the standard hydrogen electrode. Half-cells of the type shown in Table 1.7 operate as coupled pairs such that one is oxidised and the other reduced. The driving force for such a coupled reaction is the free energy change ($\Delta G^{0\prime}$) resulting from the potential difference ($\Delta E^{0\prime}$) between the two half-cells:

$$\Delta G^{0\prime} = -nF\Delta E^{0\prime}$$

where

$$\Delta E^{0\prime} = (\Delta E^{0\prime}_{\text{oxidising half-cell}} - E^{0\prime}_{\text{reducing half-cell}})$$

Some artificial redox half-cells have the attraction that they have different colours in their oxidised and reduced states. These so-called redox dyes are particularly useful for the study of enzyme reactions. This is discussed further in Section 15.2.2. Examples of redox dyes are given in Table 1.8.

Table 1.8 Standard redox potentials of some redox dyes (at 25 °C)

$E^{0\prime}$ (V)	Redox dye
−0.45	Methyl viologen
−0.36	Benzyl viologen
−0.36	Potassium ferricyanide
−0.22	2,6-Dichlorophenol indophenol (DCPIP)
−0.08	2,3,5-Triphenyltetrazolium chloride (TTC)
−0.08	Phenazine methosulphate (PMS)
−0.01	Methylene blue

1.5.2 The pH electrode and other ion-selective electrodes

Principle of the pH electrode

The pH electrode is an example of an ion-selective electrode (ISE) that responds to one specific ion in solution. Unlike the calomel and silver/silver chloride electrodes, the underlying mechanism of action of ISEs is not based on an oxidation–reduction reaction but on ion gradients. However, they all rely on the technique of potentiometry, which involves the measurement of a potential of an electrode without a current flowing.

The pH electrode consists of a thin, glass, porous membrane sealed at the end of a hard glass tube containing 0.1 M hydrochloric acid into which is immersed a silver wire coated with silver chloride. This silver/silver chloride electrode acts as an internal reference that generates a constant potential. The porous membrane is typically 0.1 mm thick, the outer and inner 10 nm consisting of a hydrated gel layer containing exchange binding sites for hydrogen or sodium ions. On the inside of the membrane the exchange sites are predominantly occupied by hydrogen ions from the hydrochloric acid, whilst on the outside the exchange sites are occupied by sodium and hydrogen ions. The bulk of the membrane is a dry silicate layer in which all exchange sites are occupied by sodium ions. Most of the coordinated ions in both hydrated layers are free to diffuse into the surrounding solution, whilst hydrogen ions in the test solution can diffuse in the opposite direction, replacing bound sodium ions in a process called ion-exchange equilibrium. Any other types of cation present in the test solution are unable to bind to the exchange sites, thus ensuring the high specificity of the electrode. Note that hydrogen ions do not diffuse across the dry glass layer but sodium ions can. Thus, effectively, the membrane consists of two hydrated layers containing different hydrogen ion activities separated by a sodium ion transport system.

The principle of operation of the pH electrode is based upon the fact that if there is a gradient of hydrogen ion activity across the membrane this will generate a potential the size of which is given by the Nernst equation (equation 1.11). In this case, however, the potential is determined solely by the hydrogen ion gradient across the membrane rather than by the ratio of oxidised to reduced species. Moreover, since the hydrogen ion activity on the inside is constant (owing to the use of 0.1 M hydrochloric acid) the observed potential is directly dependent upon

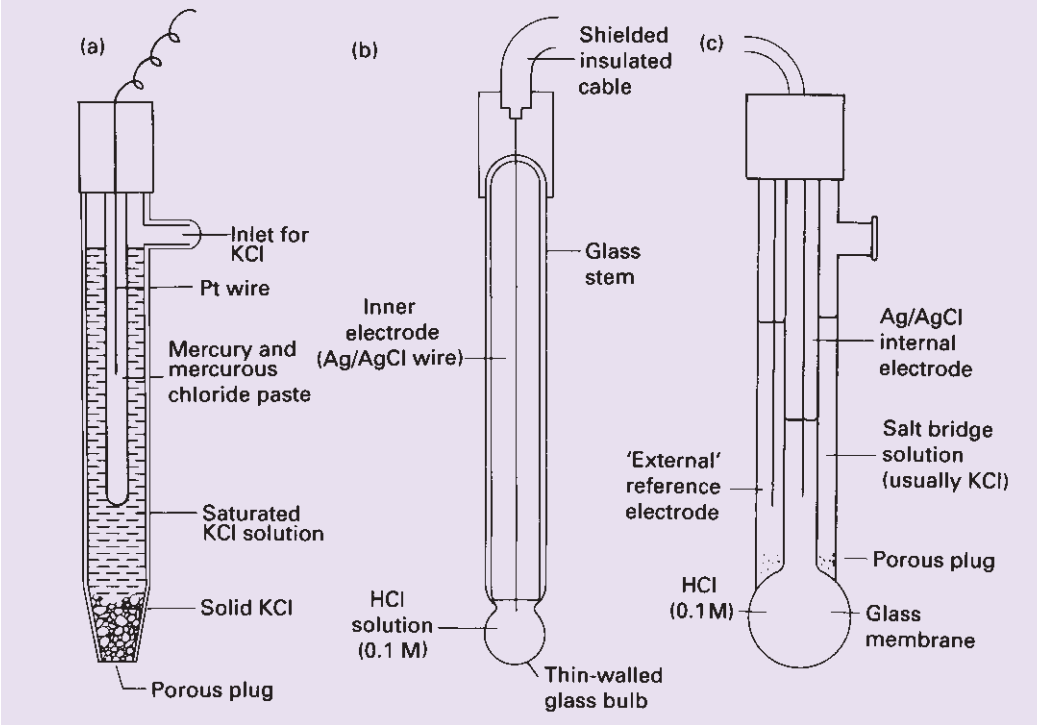

Fig. 1.1. Common electrodes: (a) calomel reference electrode; (b) glass electrode; (c) combination electrode.

the hydrogen ion activity of the test solution. It is evident from equation 1.11 that if the hydrogen ion activities on the two sides of the membrane were equal (i.e. the test solution consisted of 0.1 M hydrochloric acid), the resulting potential should be zero volts. In practice it is not, owing to a small junction or asymmetry potential (E^*) in part created by linking the glass electrode to the reference electrode. The observed potential across the membrane is therefore given by the equation:

$$E = E^* + 0.059\ \text{pH}$$

Since the precise composition of the porous membrane varies with time, so too does the asymmetry potential. This contributes to the need for the frequent recalibration of the electrode. For each 10-fold change in the hydrogen ion activity across the membrane (equivalent to a pH change of 1 in the test solution) there will be a potential difference change of 59.2 mV across the membrane. The presence of a term in the Nernst equation for the absolute temperature explains the sensitivity of pH measurements to the prevailing temperature.

The most common forms of pH electrode are the glass electrode (Fig. 1.1b) and the combination electrode (Fig. 1.1c), which contains an in-built calomel reference electrode.

Operation and calibration of a pH electrode

The glass membrane of a pH electrode is fragile and easily damaged. It is important that its surface remains hydrated and so it should be kept immersed in water when not in use. Electrodes that have been allowed to dry out should be soaked in 6 M hydrochloric acid followed by water prior to recalibration. Calibration should normally be carried out using two standard buffer solutions that span the pH range for which the electrode is to be used. Buffers of pH 4.008 (0.05 M potassium hydrogen phthalate) and 9.18 (0.01 M borax) are commonly used. The clean and blotted-dry electrode should be immersed in the buffer solution, allowed to equilibrate and the pH meter adjusted to the known pH value using the 'calibrate' knob on the instrument. The process is then repeated with the second buffer solution and any adjustment made using the 'slope' or 'temperature' knob. Recalibration may be necessary after 1–2 h of use. A well-calibrated instrument should be capable of reading to ± 0.2 pH units.

Errors in the measurement of pH

The measurement of pH is subject to several sources of error. These may be caused by a variety of factors including:

- the failure to provide adequate temperature control of the test solution;
- the failure to correctly maintain and calibrate the electrode;
- the failure to allow adequate equilibration time before the pH of the test solution is recorded;
- the presence of salts that ionise in the test solution, thereby altering the activity coefficients of the buffer components;
- the addition of organic solvents such as ethanol to the test solution, resulting in a change in the activity coefficients of the buffer components;
- the presence of proteins in the test solution that can coat the glass membrane and thereby affect the ion-exchange process at the hydrated membrane surface;
- the fact that, at pH values above 12, the low hydrogen ion concentration allows sodium ions in the test solution to replace hydrogen ions in the outer hydrated layer, in turn allowing the electrode to respond to sodium ions so that the recorded pH is lower than the actual pH. This is referred to as the sodium or alkaline error. Special glass electrodes, in which sodium ions are replaced by lithium, are available for measurements at high pH values.

Ion-selective electrodes

Electrodes exist for the measurement of many other ions including Li^+, K^+, Na^+, Ca^{2+}, Cl^- and NO_3^-. Their principle of operation is very similar to that of the pH electrode. In these cases, the permeable membrane may be made of special aluminosilicate glass or an inorganic crystal. The fluoride electrode, for example, uses a crystal of LaF_3 that responds selectively to the adsorption of fluoride ions onto its surface. A problem with most of these electrodes is their lack of absolute

specificity for the test ion. Selectivity is expressed by a selectivity coefficient that expresses the ratio of the response to the competing ions relative to that for the desired ion. The specificity of the electrode is therefore inversely proportional to the selectivity coefficient. Most commercial ISEs have both a good linear response to the desired ion and a fast response time. ISEs are used routinely in clinical biochemistry laboratories for the measurement of sodium, potassium, calcium and chloride using autoanalyser techniques (Section 1.7.2).

Gas-sensing electrodes

Electrodes responding to gases such as CO_2 and NH_3 are commercially available and are based on principles similar to those of ISEs. In the case of a CO_2 electrode the outer glass membrane of a pH electrode is sealed inside a CO_2-permeable membrane bag made of polytetrafluoroethylene (PTFE) or polyethylene containing 0.1M KCl plus a weak bicarbonate buffer. CO_2 diffuses across the membrane from the test sample until its concentration is equal across both sides. Inside the membrane it forms carbonic acid that ionises to bicarbonate and hydrogen ions. Since the bicarbonate concentration in the bag is virtually constant owing to the presence of the bicarbonate buffer, the measurement of pH can be linked directly to the concentration of CO_2 in the test sample.

Biosensors and optical sensors

Biosensors are derived from ISEs or gas-sensing electrodes by incorporating an immobilised enzyme or cell onto the surface of the electrode that then responds, via a suitable transducer (a means of converting a chemical change into an electrical or optical signal), to either the test analyte or a metabolite of it. Important biochemical examples are the glucose, urea and cholesterol biosensors. The urea biosensor consists of urease immobilised onto the surface of an ammonia-sensing electrode. The urease converts the urea in the test sample to ammonia, which is detected by the electrode:

$$CO(NH_2)_2 + H_2O \rightleftharpoons 2NH_3 + CO_2$$

The glucose and cholesterol biosensors are based on the oxidation of the test analyte (by glucose oxidase and cholesterol oxidase, respectively) and either on the amperometric measurement of the generated hydrogen peroxide by its reduction at the anode or on the direct measurement, via an oxygen electrode, of the uptake of oxygen by the oxidase:

$$\beta\text{-D-glucose} + O_2 \rightleftharpoons \text{D-glucose-1,5-lactone} + H_2O_2$$

$$\text{cholesterol} + O_2 \rightleftharpoons \text{4-cholesten-3-one} + H_2O_2$$

Optical sensors, such as that for the measurement of ATP, detect light emitted by the action of luciferase (Section 15.2.2). A photomultiplier detects and enhances the light signal, creating a very sensitive method for the measurement of ATP.

1.5.3 The oxygen electrode

Principle of operation

The utilisation of oxygen during respiration and the evolution of oxygen during photosynthesis are two of the most fundamental life processes. Early studies of these processes used the technique of manometry, which involves the measurement of gross changes in gas volume. However, the advent of the oxygen electrode has revolutionised biochemical studies of the mechanistic detail underlying the processes of oxidative phosphorylation in mitochondria and of photosynthesis in chloroplasts. The oxygen electrode is an electrochemical cell containing a platinum cathode and a silver anode, both of which are separated from the test solution by an oxygen-permeable membrane. A polarising potential of 0. 6 V is applied across the electrodes so that oxygen near the cathode surface is reduced by electrons, whilst at the anode electrons are released as a result of an oxidation process:

At the cathode $O_2 + 4H^+ + 4e^- \rightleftharpoons 2H_2O$ reduction

At the anode $4Ag + 4Cl^- \rightleftharpoons 4AgCl + 4e^-$ oxidation

The reductive removal of the oxygen at the cathode surface allows more oxygen in the test solution to diffuse across the permeable membrane and for the process of reduction at the cathode to continue. This generates a current, the size of which is directly proportional to the amount of oxygen (referred to as the oxygen tension) in the test solution. The oxygen electrode is therefore based on the process of amperometry (the measurement of a current flowing through an electrode at a constant potential).

It is important to ensure that the diffusion of oxygen from the bulk of the test solution to the membrane surface does not become a limiting factor for the generation of the current. To avoid this problem, the test solution must be stirred efficiently. The oxygen electrode gives a continuous record of the oxygen tension in the test solution, for example during oxidative phosphorylation (Fig. 1.2). Prior to each experiment the electrode has to be calibrated by the use of air-saturated water and oxygen-depleted water (usually achieved by the addition of a crystal of sodium dithionite to the water). The concentration of oxygen in air-saturated water at various temperatures is recorded in tables of physical constants.

Oxygen electrodes are available commercially in many forms. One of the most widely used is the Rank electrode shown diagrammatically in Fig. 1.3. The reaction vessel has a volume of about 3 cm^3. The oxygen-permeable membrane may be made of Teflon™, Cellophane™ or silicone rubber. All membranes are sensitive to contamination particularly by proteins. The anode and cathode are connected to each other via a solution of potassium chloride.

Applications of the oxygen electrode

The use of an oxygen electrode is the method of choice for the study of any biochemical process in which there is the uptake or evolution of oxygen. The method has proved particularly successful in the study of respiratory control, the effect of

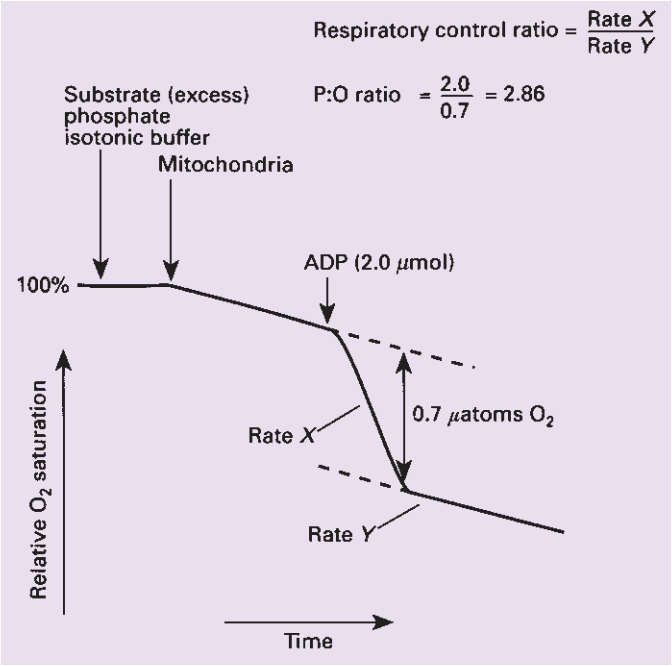

Fig. 1.2. A typical experimental trace of oxygen consumption for intact mitochondria obtained using an oxygen electrode.

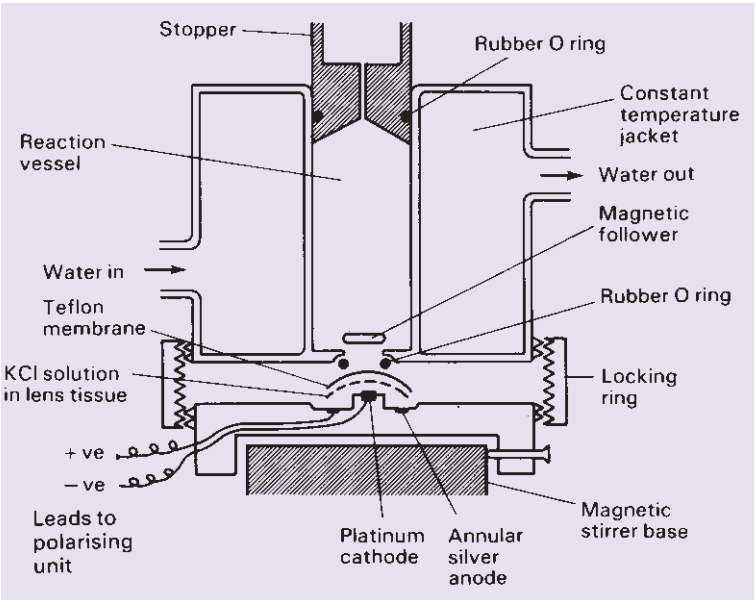

Fig. 1.3. Section through a Rank oxygen electrode.

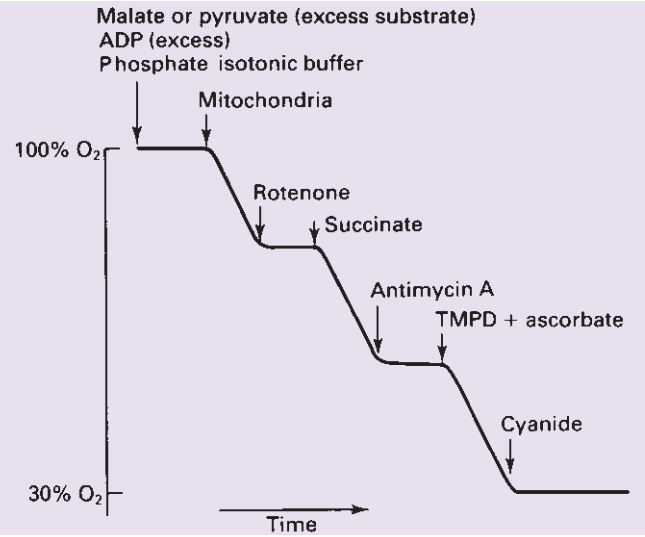

Fig. 1.4. Oxygen electrode trace showing the effect of inhibitors of electron transport and electron donors on mitochondrial respiration TMPD, tetramethylphenylenediamie.

inhibitors on mitochondrial respiration, and the measurement of P:O ratios in oxidative and photosynthetic phosphorylation. Fig. 1.2 records oxygen consumption in intact mitochondria. The trace shows slow oxygen consumption by the mitochondria until ADP is added. This stimulates oxygen uptake by the promotion of ATP production (rate *X*) until all the ADP has been consumed, at which point the rate declines to *Y*. The ratio of *X* to *Y* is a measure of the respiratory control ratio. From knowledge of the ADP added and the oxygen consumed the P:O ratio can be calculated. The sites of action of respiratory inhibitors on the two processes and the way the inhibition can be overcome by electron donors can also be studied, as shown in Fig. 1.4.

Variants of the Rank electrode are available that allow the study of oxygen exchange in the gas phase rather than in aqueous solution.

1.5.4 Electrochemical study of single cells

In recent years the miniaturisation of electrodes has stimulated studies of quantitative cellular analysis. Microelectrodes with a tip diameter in the range 1–5 μm, response time of a few microseconds and capability of measuring at the level of zeptomoles (zmol, i.e. 10^{-21} mol, representing approximately 600 molecules) have been developed that have enabled the dynamics of cellular processes to be investigated. The electrodes are most commonly made of carbon fibre and have been constructed in disc-shaped and cylindrical geometries. Microelectrodes of the ion-selective type have been used to monitor intracellular levels of H^+, Li^+, Ca^{2+} and Mg^{2+}, whilst platinum microelectrodes have been used to study compounds that readily undergo oxidation or reduction at the surface of the electrode. Such compounds include dopamine, enabling its release from single nerve cells to be

studied. Microelectrodes have also been used to study the dynamics of exocytosis. Studies with cultured bovine medullary chromaffin cells, for example, have demonstrated that an average of 5–10 attomoles (i.e. 10^{-18} moles) of serotonin are released per exocytotic event.

1.6 QUANTITATIVE BIOCHEMICAL MEASUREMENTS

1.6.1 Analytical considerations and experimental error

Many biochemical investigations involve the quantitative determination of the concentration and/or amount of a particular component (the analyte) present in a test sample. For example, in studies of the mode of action of enzymes, transmembrane transport and cell signalling, the measurement of a particular reactant or product is investigated as a function of a range of experimental conditions and the data used to calculate kinetic or thermodynamic constants. These in turn are used to deduce details of the mechanism of the biological process taking place. Irrespective of the experimental rationale for undertaking such quantitative studies, all quantitative experimental data must first be questioned and validated in order to give credibility to the derived data and the conclusions that can be drawn from it. This requires that the experimental data be assessed and confirmed as an acceptable estimate of the 'true' values by the application of one or more standard statistical tests. Evidence of the validation of quantitative data by the application of such tests is a standard requirement by the editors of peer-refereed journals for the acceptance for publication of draft research papers. The following sections will address the theoretical and practical considerations behind these statistical tests.

The test sample

The test material for quantitative analysis may be of a widely diverse nature. Examples include a preparation of a purified protein or nucleic acid, an organelle preparation, a cell suspension or homogenate, a tissue homogenate, a sample of physiological fluid such as urine, serum, plasma or whole blood, or the eluant from a chromatographic column. The matrix (the nature of medium in which the analyte is present such as water, saline, serum, urine) will influence the process of sampling. Sampling is the process by which a representative portion is taken for analysis. Homogeneous, aqueous test solutions present no problem in this respect but if the sample is viscous the accurate sampling of a given volume is more difficult. If the test material is heterogeneous it is particularly difficult to ensure that the sample taken for analysis is representative of the whole. For example, it might be appropriate to grind plant material in a pestle and mortar to ensure the representation of the selected sample for the analysis. Alternatively, it may be necessary to undertake some form of preliminary extraction so that the selected sample is in a form suitable for analysis. Possible extraction techniques include liquid extraction, in which the test material is continuously extracted with an organic solvent such as dichloromethane or chloroform, and solid-phase extraction, which is a form of

adsorption or ion-exchange chromatography. Some potential test samples may be too concentrated for direct analysis and in such cases the question of a suitable diluent arises. For example, if the concentration of an analyte in serum or plasma is too high, dilution of the sample with water would automatically alter the activity as well as the concentration of the analyte and dilution with a 'blank' serum or plasma sample would not be possible.

Selecting an analytical method

The nature of the quantitative analysis to be carried out will require a decision to be taken on the analytical technique to be employed. A variety of methods may be capable of achieving the desired analysis and the decision to select one may depend on a variety of issues. These include:

- the availability of specific pieces of apparatus;
- the precision, accuracy and detection limits of the competing methods;
- the precision, accuracy and detection limit acceptable for the particular analysis;
- the number of other compounds present in the sample that may interfere with the analysis;
- the potential cost of the method (particularly important for repetitive analysis);
- the possible hazards inherent in the method and the appropriate precautions needed to minimise risk;
- the published literature method of choice;
- personal preference.

The most common biochemical quantitative analytical methods are visible, ultraviolet and fluorimetric spectrophotometry, chromatographic techniques such as high performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC) coupled to spectrophotometry or mass spectrometry, ion-selective electrodes and immunological methods such as the enzyme-linked immunosorbent assay (ELISA). Once a method has been selected it must be developed and/or validated using the approaches discussed in the following sections. If it is to be used over a prolonged period of time, measures will need to be put in place to ensure that there is no drift in response. This normally entails an internal quality control approach using reference test samples covering the analytical range that are measured each time the method is applied to test samples. Any deviation from the known values for these reference samples will require the whole batch of test samples to be reassayed.

The nature of experimental errors

Every quantitative measurement has some uncertainty associated with it. This uncertainty is referred to as the experimental error, which is a measure of the difference between the 'true' value and the experimental value. The 'true' value normally remains unknown except in cases where a standard sample (i.e. one of known composition) is being analysed. In other cases it has to be estimated from

the analytical data by the methods that will be discussed later. The consequence of the existence of experimental errors is that the measurements recorded can be accepted with a high, medium or low degree of confidence depending upon the sophistication of the technique employed, but seldom, if ever, with absolute certainty.

Experimental error may be of two kinds: systematic error and random error.

Systematic error (also called determinate error)

Systematic errors are consistent errors that can be identified and either eliminated or reduced. They are most commonly caused by a fault or inherent limitation in the apparatus being used but may also be influenced by poor experimental design. Common causes include the misuse of manual or automatic pipettes, the incorrect preparation of stock solutions, and the incorrect calibration and use of pH meters. They may be constant (i.e. have a fixed value irrespective of the amount of test analyte present in the test sample under investigation) or proportional (i.e. the size of the error is dependent upon the amount of test analyte present). Thus the overall effect of the two types in a given experimental result will differ. Both of these types of systematic error have three common causes:

- *Analyst error:* This is best minimised by good training and/or by the automation of the method.
- *Instrument error:* This may not be eliminable and hence alternative methods should be considered. Instrument error may be electronic in origin or may be linked to the matrix of the sample.
- *Method error:* This can be identified by comparison of the experimental data with that obtained by the use of alternative methods.

Identification of systematic errors

Systematic errors are always reproducible and may be positive or negative, i.e. they increase or decrease the experimental value relative to the 'true' value. The crucial characteristic, however, is that their cause can be identified and corrected. There are four common means of identifying this type of error:

- *Use of a blank sample:* This is a sample that you know contains none of the analyte under test so that if the method gives a non-zero answer then it must be responding in some unintended way. The use of blank samples is difficult in cases where the matrix of the test sample is complex, for example serum.
- *Use of a standard reference sample:* This is a sample of the test analyte of known composition so the method under evaluation must reproduce the known answer.
- *Use of an alternative method:* If the test and alternative methods give different results for a given test sample then at least one of the methods must have an in-built flaw.
- *Use of an external quality assessment sample:* This is a standard reference sample that is analysed by other investigators based in different laboratories employing the same or different methods. Their results are compared and any

differences in excess of random errors (see below) identify the systematic error for each analyst. The use of external quality assessment schemes is standard practice in clinical biochemistry laboratories (Section 1.7.3).

Random error (also called indeterminate error)

Random errors are caused by unpredictable and often uncontrollable inaccuracies in the various manipulations involved in the method. These errors may be variably positive or negative and are caused by factors such as difficulty in the process of sampling, random electrical 'noise' in an instrument, or by the analyst being inconsistent in the operation of the instrument or of recording readings from it.

Standard operating procedures

The minimisation of both systematic and random errors is essential in cases where the analytical data are used as the basis for a crucial diagnostic or prognostic decision as is common, for example, in routine clinical biochemical investigations and in the development of new drugs. In such cases it is normal for the analyses to be conducted in accordance with standard operating procedures (SOPs) that define in full detail the quality of the reagents, the preparation of standard solutions, the calibration of instruments and the methodology of the actual analytical procedure that must be followed.

1.6.2 Assessment of the performance of an analytical method

All analytical methods can be characterised by a number of performance indicators that define how the selected method performs under specified conditions. Knowledge of these performance indicators allows the analyst to decide whether or not the method is acceptable for the particular application. The major performance indicators are:

- *Precision (also called imprecision and variability):* This is a measure of the reproducibility of a particular set of analytical measurements on the same sample of test analyte. If the replicated values agree closely with each other, the measurements are said to be of high precision (or low imprecision). In contrast, if the values diverge, the measurements are said to be of poor or low precision (or high imprecision). In analytical biochemical work the normal aim is to develop a method that has as high a precision as possible within the general objectives of the investigation. However, precision commonly varies over the analytical range (see below) and over periods of time. As a consequence, precision may be expressed as either within batch or between batch. Within-batch precision is the variability when the same test sample is analysed repeatedly during the same batch of analyses on the same day. Between-batch precision is the variability when the same test sample is analysed repeatedly during different batches of analyses over a period of time. Since there is more opportunity for the analytical conditions to change for the assessment of between-batch precision, it is the higher of the two types

of assessment. Results that are of high precision may nevertheless be a poor estimate of the 'true' value (i.e. of low accuracy or high bias) because of the presence of unidentified errors. Methods for the assessment of precision of a data set are discussed below. The term imprecision is preferred in particular by clinical biochemists, since they believe that it best describes the variability that occurs in replicated analyses.

- *Accuracy (also called bias and inaccuracy):* This is the difference between the mean of a set of analytical measurements on the same sample of test analyte and the 'true' value for the test sample. As previously pointed out, the 'true' value is normally unknown except in the case of standard measurements. In other cases accuracy has to be assessed indirectly by use of an internationally agreed reference method and/or by the use of external quality assessment schemes (see above) and/or by the use of population statistics that are discussed below.
- *Detection limit (also called sensitivity):* This is the smallest concentration of the test analyte that can be distinguished from zero with a defined degree of confidence. Concentrations below this limit should simply be reported as 'less than the detection limit'. All methods have their individual detection limits for a given analyte and this may be one of the factors that influence the choice of a specific analytical method for a given study. Thus the Bradford, Lowry and bicinchoninic acid methods for the measurements of proteins have detection limits of 20, 10 and 0. 5 μg protein cm^{-3}, respectively. In clinical biochemical measurements, sensitivity is often defined as the ability of the method to detect the analyte without giving false negatives (Section 1.7.1).
- *Analytical range:* This is the range of concentrations of the test analyte that can be measured reproducibly, the lower end of the range being the detection limit. In most cases the analytical range is defined by an appropriate calibration curve (Section 1.6.6). As previously pointed out, the precision of the method may vary across the range.
- *Analytical specificity (also called selectivity):* This is a measure of the extent to which other substances that may be present in the sample of test analyte may interfere with the analysis and therefore lead to a falsely high or low value. A simple example is the ability of a method to measure glucose in the presence of other hexoses such as mannose and galactose. In clinical biochemical measurements, selectivity is an index of the ability of the method to give a consistent negative result for known negatives (Section 1.7.1).
- *Analytical sensitivity:* This is a measure of the change in response of the method to a defined change in the quantity of analyte present. In many cases analytical sensitivity is expressed as the slope of a linear calibration curve.
- *Robustness:* This is a measure of the ability of the method to give a consistent result in spite of small changes in experimental parameters such as pH, temperature, or amount of reagents added. For routine analysis, the robustness of a method is an important practical consideration.

These performance indicators are established by the use of well-characterised test and reference analyte samples. The order in which they are evaluated will depend on the immediate analytical priorities, but initially the three most important may be specificity, detection limit and analytical range. Once a method is in routine use, the question of assuring the quality of analytical data by the implementation of quality assessment procedures comes into play (Section 1.7.3).

1.6.3 Assessment of precision

After a quantitative study has been completed and an experimental value for the amount and/or concentration of the test analyte in the test sample obtained, the experimenter must ask the question 'How confident can I be that my result is an acceptable estimate of the "true" value?' (i.e. is it accurate?). An additional question may be 'Is the quality of my analytical data comparable with that in the published scientific literature for the particular analytical method?' (i.e. is it precise?). Once the answers to such questions are known, a result that has a high probability of being correct can be accepted and used as a basis for the design of further studies, whilst a result that is subject to unacceptable error can be rejected. Unfortunately it is not possible to assess the precision of a single quantitative determination. Rather, it is necessary to carry out analyses in replicate (i.e. the experiment is repeated several times on the same sample of test analyte) and to subject the resulting data set to some basic statistical tests.

If a particular experimental determination is repeated numerous times and a graph constructed of the number of times a particular result occurs against its value, it is normally bell shaped, with the results clustering symmetrically about a mean value. This type of distribution is called a Gaussian or normal distribution. In such cases the precision of the data set is a reflection of random error. However, if the plot is skewed to one side of the mean value, then systematic errors have not been eliminated. Assuming that the data set is of the normal distribution type, there are three statistical parameters that can be used to quantify precision.

Standard deviation, coefficient of variation and variance – measures of precision

These three statistical terms are different ways of expressing the scatter of the values within a data set about the mean (calculated by summing their total value and dividing by the number of individual values). Each term has its individual merit. In all three cases the term is actually measuring the width of the normal distribution curve such that the narrower the curve the smaller the value of the term and the higher the precision of the analytical data set.

The standard deviation (s) of a data set is calculated by use of equation 1.12 or 1.13:

$$s = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}} \qquad (1.12)$$

$$s = \sqrt{\frac{\sum x_i^2 - \left[\left(\sum x_i\right)^2 / n\right]}{n - 1}} \tag{1.13}$$

$(x_i - \bar{x})$ is the difference between an individual experimental value (x_i) and the calculated mean ($\bar{x}$) of the individual values. Since these differences may be positive or negative, and since the distribution of experimental values about the mean is symmetrical, if they were simply added together they would cancel each other out. The differences are therefore squared to give consistent positive values. To compensate for this, the square root of the resulting calculation has to be taken to obtain the standard deviation.

Standard deviation (SD) has the same units as the actual measurements and this is one of its attractions. The mathematical nature of a normal distribution curve is such that 68.2% of the area under the curve (and hence 68.2% of the individual values within the data set) is within one standard deviation either side of the mean, 95.5% of the area under the curve is within two standard deviations and 99.7% within three standard deviations. Exactly 95% of the area under the curve falls between the mean and 1.96 standard deviations. The precision (or imprecision) of a data set is commonly expressed as ± 1 SD of the mean.

The term $(n - 1)$ is called the degrees of freedom of the data set and is an important variable. The initial number of degrees of freedom possessed by a data set is equal to the number of results (n) in the set. However, when another quantity characterising the data set, such as the mean or standard deviation, is calculated, the number of degrees of freedom of the set is reduced by 1 and by 1 again for each new derivation made. Many modern calculators and computers include programs for the calculation of standard deviation. However, some use variants of equation 1.12 in that they use n as the denominator rather than $n - 1$ as the basis for the calculation. If n is large, greater than 30 for example, then the difference between the two calculations is small, but if n is small, and certainly if it is less than 10, the use of n rather than $n - 1$ will significantly underestimate the standard deviation. This may lead to false conclusions being drawn about the precision of the data set. Thus, for most analytical biochemical studies, it is imperative that the calculation of the standard deviation is based on the use of $n - 1$.

The coefficient of variation (CV) (also known as relative standard deviation) of a data set is the standard deviation expressed as a percentage of the mean, as shown in equation 1.14.

$$\text{CV} = \frac{s\,100\%}{\bar{x}} \tag{1.14}$$

Since the mean and standard deviation have the same units, the coefficient of variation is simply a percentage. This independence of the unit of measurement allows methods based on different units to be compared.

The variance of a data set is the mean of the squares of the differences between each value and the mean of the values. It is also the square of the standard deviation, hence the symbol s^2. It has units that are the square of the original units and

this makes it appear rather cumbersome, which explains why standard deviation and coefficient of variation are the preferred ways of expressing the variability of data sets. The importance of variance will be evident in later discussions of the ways of making a statistical comparison of two data sets.

To appreciate the relative merits of the standard deviation and coefficient of variation as measures of precision, consider the following scenario. Suppose that two serum samples, A and B, were each analysed 20 times for serum glucose by the glucose oxidase method (Section 15.2.5) such that sample A gave a mean value of 2.00 mM with a standard deviation of ±0.10 mM and sample B a mean of 8.00 mM and a standard deviation of ± 0.41 mM. On the basis of the standard deviation values it might be concluded that the method had given a better precision for sample A than for sample B. However, this ignores the absolute values of the two samples. If this is taken into account by calculating the coefficient of variation, the two values are 5.0% and 5.1%, respectively, showing that the method had given the same precision for both samples. This illustrates the fact that the standard deviation is an acceptable assessment of precision for a given data set but, if it is necessary to compare the precision of two or more data sets, particularly ones with different mean values, then the coefficient of variation should be used. The majority of well-developed analytical methods have a coefficient of variation within the analytical range of less than 5% and many, especially automated methods, of less than 2%.

1.6.4 Assessment of accuracy

Population statistics

Whilst standard deviation and coefficient of variation give a measure of the precision of the data set they do not quantify how well the mean of the data set approaches the 'true' value. To address this issue it is necessary to introduce the concepts of population statistics and confidence level and confidence interval. If a data set is made up of a very large number of individual values so that n is a large number, then the mean of the set would be equal to the population mean mu (μ) and the standard deviation would equal the population standard deviation sigma (σ). Note that Greek letters represent the population parameters and the roman alphabet the sample parameters. These two population parameters are the best estimates of the 'true' values, since they are based on the largest number of individual measurements, so the influence of random errors is minimised. In practice the population parameters are seldom measured for obvious practicality reasons and the sample parameters have a larger uncertainty associated with them. The uncertainty of the sample mean deviating from the population mean decreases in proportion to the reciprocal of the square root of the number of values in the data set, i.e. $1/\sqrt{n}$. Thus, to decrease the uncertainty by a factor of 2, the number of experimental values would have to be increased 4-fold and for a factor of 10 the number of measurements would need to be increased 100-fold. The nature of this relationship again emphasises the importance of evaluating the acceptable degree

Example 5 ASSESSMENT OF THE PRECISION OF AN ANALYTICAL DATA SET

Question

Five measurements of the fasting serum glucose concentration were made on the same sample taken from a diabetic patient. The values obtained were 2.3, 2.5, 2.2, 2.6 and 2.5 mM. Calculate the precision of the data set.

Answer

Precision is normally expressed either as one standard deviation of the mean or as the coefficient of variation of the mean. These statistical parameters therefore need to be calculated.

Mean

$$\bar{x} = \frac{2.2 + 2.3 + 2.5 + 2.5 + 2.6}{5} = 2.42 \text{ mM}$$

Standard deviation

Using both equations 1.12 and 1.13 to calculate the value of s:

x_i	$x_i - \bar{x}$	$(x_i - \bar{x})^2$	x_i^2
2.2	−0.22	0.0484	4.84
2.3	−0.12	0.0144	5.29
2.5	+0.08	0.0064	6.25
2.5	+0.08	0.0064	6.25
2.6	+0.18	0.0324	6.75
Σx_i 12.1	Σ0.00	Σ0.1080	Σ29.39

Using equation 1.12

$$s = \sqrt{(0.108/4)} = 0.164 \text{ mM}$$

Using equation 1.13

$$s = \sqrt{\frac{29.39 - (12.1)^2/5}{4}} = \sqrt{\frac{29.39 - 29.28}{4}} = 0.166 \text{ mM}$$

Coefficient of variation

Using equation 1.9

$$\text{CV} = \frac{0.165 \times 100\%}{2.42}$$

$$= 6.82\%$$

Discussion

In this case it is easier to appreciate the precision of the data set by considering the coefficient of variation: 6.82% is moderately high for this type of analysis. Automation of the method would certainly reduce it by at least half. Note that it is legitimate to quote the answers to these calculations to one more digit than was present in the original data set. In practice, it is advisable to carry out the statistical analysis on a far larger data set than that presented in this example.

of uncertainty of the experimental result before the design of the experiment is completed and the practical analysis begun. Modern automated analytical instruments recognise the importance of multiple results by facilitating repeat analyses at maximum speed. It is good practice to report the number of measurements on which the mean and standard deviation are based, as this gives a clear indication of the quality of the calculated data.

Confidence intervals, confidence level and the Student's *t* factor

Accepting that the population mean is the best estimate of the 'true' value, the question arises 'How can I relate my experimental sample mean to the population mean?' The answer is by using the concept of confidence. Confidence level expresses the level of confidence, expressed as a percentage, that can be attached to the data. Its value has to be set by the experimenter to achieve the objectives of the study. Confidence interval is a mathematical statement relating the sample mean to the population mean. A confidence interval gives a range of values about the sample mean within which there is a given probability (determined by the confidence level) that the population mean lies. The relationship between the two means is expressed in terms of the standard deviation of the data set, the square root of the number of values in the data set and a factor known as Student's *t* (equation 1.15):

$$\mu = \bar{x} \pm \frac{t\,s}{\sqrt{n}} \tag{1.15}$$

where $\bar{x}$ is the measured mean, μ is the population mean, s is the measured standard deviation, n is the number of measurements, and t is the Student's t factor. The term $s/\sqrt{n}$ is known as the standard error of the mean and is a measure of the reliability of the sample mean as a good estimate of the population mean.

Confidence level can be set at any value up to 100%. For example, it may be that a confidence level of only 50% would be acceptable for a particular experiment. However, a 50% level means that that there is a 1 in 2 chance that the sample mean is not an acceptable estimate of the population mean. In contrast, the choice of a 95% or 99% confidence level would mean that there was only a 1 in 20 or a 1 in 100 chance, respectively, that the best estimate had not been achieved. In practice, most analytical biochemists choose a confidence level in the range 90–99% and most commonly 95%.

Student's *t* is a way of linking probability with the size of the data set and is used in a number of statistical tests. Student's *t* values for varying numbers in a data set (and hence with varying degrees of freedom) at selected confidence levels are available in statistical tables. Some values are shown in Table 1.9. The numerical value of *t* is equal to the number of standard errors of the mean that must be added and subtracted from the mean to give the confidence interval at a given confidence level. Note that, as the sample size (and hence the degrees of freedom) increases, the confidence levels converge. When n is large and if we wish to calculate the 95% confidence interval, the value of t approximates to 1.96 and

Table 1.9 Values of Student's *t*

Degrees of freedom	Confidence level (%)					
	50	90	95	98	99	99.9
2	0.816	2.920	4.303	6.965	9.925	31.598
3	0.765	2.353	3.182	4.541	5.841	12.924
4	0.741	2.132	2.776	3.747	4.604	8.610
5	0.727	2.015	2.571	3.365	4.032	6.869
6	0.718	1.943	2.447	3.143	3.707	5.959
7	0.711	1.895	2.365	2.998	3.500	5.408
8	0.706	1.860	2.306	2.896	3.355	5.041
9	0.703	1.833	2.262	2.821	3.250	4.798
10	0.700	1.812	2.228	2.764	3.169	4.587
15	0.691	1.753	2.131	2.602	2.947	4.073
20	0.687	1.725	2.086	2.528	2.845	3.850
30	0.683	1.697	2.042	2.457	2.750	3.646

some texts quote equation 1.15 in this form. The term Student's *t* factor may give the impression that it was devised specifically with students' needs in mind. In fact 'Student' was the pseudonym of a statistician, by the name of W. S. Gossett, who in 1908 first devised the term and who was not permitted by his employer to publish his work under his own name.

Criteria for the rejection of outlier experimental data – *Q*-test

A very common problem in quantitative biochemical analysis is the need to decide whether or not a particular result is an outlier and should therefore be rejected before the remainder of the data set is subjected to statistical analysis. It is important to identify such data as they have a disproportionate effect on the calculation of the mean and standard deviation of the data set. When faced with this problem, the first action should be to check that the suspected outlier is not due to a simple experimental or mathematical error. Once the suspect figure has been confirmed its validity is checked by application of Dixon's *Q*-test. Like other tests to be described later, the test is based on a null hypothesis, namely that there is no difference in the values being compared. If the hypothesis is proved to be correct then the suspect value cannot be rejected. The suspect value is used to calculate an experimental rejection quotient, Q_{exp}. Q_{exp} is then compared with tabulated critical rejection quotients, Q_{table}, for a given confidence level and the number of experimental results (Table 1.10). If Q_{exp} is less than Q_{table} the null hypothesis is confirmed and the suspect value should not be rejected, but if it is greater then the value can be rejected. The basis of the test is the fact that, in a normal distribution, 95.5% of the values are within the range of two standard deviations of the mean. In setting limits for the acceptability or rejection of data, a compromise has to be made on the confidence level chosen. If a high confidence level is chosen, the limits of acceptability are set wide and therefore there is a risk of accepting values

Example 6 ASSESSMENT OF THE ACCURACY OF AN ANALYTICAL DATA SET

Question

Calculate the confidence intervals at the 50%, 95% and 99% confidence levels of the fasting serum glucose concentrations given in Example 5.

Answer

Accuracy in this type of situation is expressed in terms of confidence intervals that express a range of values over which there is a given probability that the 'true' values lies.

As previously calculated, $\bar{x} = 2.42$ mM and $s = 0.16$ mM. Inspection of Table 1.9 reveals that, for 4 degrees of freedom (the number of experimental values minus 1) and a confidence level of 50%, $t = 0.741$ so that the confidence interval for the population mean is given by:

$$\text{Confidence interval} = 2.42 \pm \frac{(0.741)(0.16)}{\sqrt{5}}$$

$$= 2.42 \pm 0.05 \text{ mM}$$

For the 95% confidence level and the same number of degrees of freedom, $t = 2.776$, hence the confidence interval for the population mean is given by:

$$\text{Confidence interval} = 2.42 \pm \frac{(2.776)(0.16)}{\sqrt{5}}$$

$$= 2.42 \pm 0.20 \text{ mM}$$

For the 99% confidence level and the same number of degrees of freedom, $t = 4.604$; hence the confidence interval for the population mean is given by:

$$\text{Confidence interval} = 2.42 \pm \frac{(4.604)(0.16)}{\sqrt{5}}$$

$$= 2.42 \pm 0.33 \text{ mM}$$

Discussion

These calculations show that there is a 50% chance that the population mean lies in the range 2.37–2.47 mM, a 95% chance that the population mean lies within the range 2.22–2.62 mM and a 99% chance that it lies in the range 2.09–2.75 mM. Note that as the confidence level increases the range of potential values for the population mean also increases. You can calculate for yourself that if the mean and standard deviation had been based on 20 measurements (i.e. a 4-fold increase in the number of measurements) then the 50% and 95% confidence intervals would have been reduced to 2.42 ± 0.02 mM and 2.42 ± 0.07mM, respectively. This re-emphasises the beneficial impact of multiple experimental determinations but at the same time highlights the need to balance the value of multiple determinations against the accuracy with which the experimental mean is required within the objectives of the individual study.

that are subject to error. If the confidence level is set too low, the acceptability limits will be too narrow and therefore there will be a risk of rejecting legitimate data. In practice a confidence level of 90% or 95% is most commonly applied. The Q_{table} values in Table 1.10 are based on a 95% confidence level.

Table 1.10 Values of *Q* for the rejection of outliers

Number of observations	*Q* (95% confidence)
4	0.83
5	0.72
6	0.62
7	0.57
8	0.52

The calculation of Q_{exp} is based upon equation 1.16, which requires the calculation of the separation of the questionable value from the nearest acceptable value (gap) coupled with knowledge of the range covered by the data set:

$$Q_{exp} = \frac{x_n - x_{n-1}}{x_n - x_1} = \frac{\text{gap}}{\text{range}} \tag{1.16}$$

where x is the value under investigation in the series $x_1, x_2, x_3, \ldots, x_{n-1}, x_n$.

Example 7 IDENTIFICATION OF AN OUTLIER EXPERIMENTAL RESULT

Question

If the data set in Example 6 contained an addition value of 3.0 mM, could this value be regarded as an outlier point at the 95% confidence level?

Answer

From equation 1.16

$$Q_{exp} = \frac{3.0 - 2.6}{3.0 - 2.2} = \frac{0.4}{0.8} = 0.5$$

Using Table 1.11 for six data points, $Q_{table} = 0.62$.

Since Q_{exp} is smaller than Q_{table} the point should not be rejected as there is a more than 5% chance that it is part of the same data set as the other five values. It is easy to show that an additional data point of 3.3 rather than 3.0 mM would give a Q_{exp} of 0.64 and could be rejected.

1.6.5 Validation of an analytical method – the use of *t*-tests

A *t*-test in general is used to address the question of whether or not two data sets have the same mean. Both data sets need to have a normal distribution and equal variances. There are three types:

- *Unpaired t-test:* Used to test whether two data sets have the same mean.
- *Paired t-test:* Used to test whether two data sets have the same mean, where each value in one set is paired with a value in the other set.
- *One-sample t-test:* Used to test whether the mean of a data set is equal to a particular value.

Each test is based on a null hypothesis, which is that there is no difference between the means of the two data sets. The tests measures how likely the hypothesis is to be true. The attraction of such tests is that they are easy to carry out and interpret.

Analysis of a standard solution – one-sample *t*-test

Once the choice of the analytical method to be used for a particular biochemical assay has been made, the normal first step is to carry out an evaluation of the method in the laboratory. This evaluation entails the replicated analysis of a known standard solution of the test analyte and the calculation of the mean and standard deviation of the resulting data set. The question is then asked 'Does the mean of the analytical results agree with the known value of the standard solution within experimental error?' To answer this question a *t*-test is applied.

In the case of the analysis of a standard solution the calculated mean and standard deviation of the analytical results are used to calculate a value of the Student's t (t_{calc}) using equation 1.17. It is then compared with table values of t (t_{table}) for the particular degrees of freedom of the data set and at the required confidence level (Table 1.10).

$$t_{calc} = \frac{(\text{known value} - \bar{x})\sqrt{n}}{s} \tag{1.17}$$

These table values of t represent critical values that mark the border between different probability levels. If t_{calc} is greater than t_{table} the analytical results are deemed not to be from the same data set as the known standard solution at the selected confidence level. In such cases the conclusion is therefore drawn that the analytical results do not agree with the standard solution and hence that there are unidentified errors in them. There would be no point in applying the analytical method to unknown test analyte samples until the problem has been resolved.

Comparing two competitive analytical methods – unpaired *t*-test

In quantitative biochemical analysis it is frequently helpful to compare the performance of two alternative methods of analysis in order to establish whether or not they give the same quantitative result within experimental error. To address this need, each method is used to analyse the same test sample using replicated analysis. The mean and standard deviation for each set of analytical data is then calculated and a Student's *t*-test applied. In this case the *t*-test measures the overlap between the data sets such that the smaller the value of t_{calc} the greater the overlap between the two data sets. This is an example of an unpaired *t*-test.

In using the tables of critical *t*-values, the relevant degrees of freedom is the sum of the number of values in the two data sets (i.e. $n_1 + n_2$) minus 2. The larger the number of degrees of freedom the smaller the value of t_{calc} needs to be to exceed the critical value at a given confidence level. The formulae for calculating t_{calc} depend on whether or not the standard deviations of the two data sets are the same. This is often obvious by inspection, the two standard deviations being similar. However,

Example 8 VALIDATING AN ANALYTICAL METHOD

Question

A standard solution of glucose is known to be 5.05 mM. Samples of it were analysed by the glucose oxidase method (for details see Section 15.2.5) that was being used in the laboratory for the first time. A calibration curve obtained using least mean square linear regression was used to calculate the concentration of glucose in the test sample. The following experimental values were obtained: 5.12, 4.96, 5.21, 5.18, 5.26 mM. Does the experimental data set for the glucose solution agree with the known value within experimental error?

Answer

It is first necessary to calculate the mean and standard deviation for the set and then to use it to calculate a value for Student's *t*.

Applying equations 1.12 and 1.13 to the data set gives $\bar{x} = 5.15$ mM and $s = \pm 0.1$ mM.

Now applying equation 1.17 to give t_{calc}:

$$t_{calc} = \frac{(5.05 - 5.15)\sqrt{5}}{0.1} = 2.236$$

Note that the negative difference between the two mean values in this calculation is ignored. From Table 1.9 at the 95% confidence level with 4 degrees of freedom, $t_{table} = 2.776$. t_{calc} is therefore less than t_{table} and the conclusion can be drawn that measured mean value does agree with the known value. Using equation 1.14, the coefficient of variation for the measured values can be calculated to be 1.96%.

if in doubt, an *F*-test, named after Fisher who introduced it, can be applied. An *F*-test is based on the null hypothesis that there is no difference between the two variances. The test calculates a value for F (F_{calc}), which is the ratio of the larger of the two variances to the smaller variance. It is then compared with critical *F*-values (F_{table}) available in statistical tables or computer packages (Table 1.11). If the calculated value of F is less than the table value, the null hypothesis is proved and the two standard deviations are considered to be similar. If the two variances are of the same order, then equations 1.18 and 1.19 are used to calculate t_{calc} for the two data sets. If not, equations 1.20 and 1.21 are used.

$$t_{calc} = \frac{\bar{x}_1 - \bar{x}_2}{S_{pooled}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \tag{1.18}$$

$$S_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{n_1 + n_2 - 2}} \tag{1.19}$$

$$t_{calc} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s_1^2/n_1) + (s_2^2/n_2)}} \tag{1.20}$$

$$\text{Degrees of freedom} = \left\{ \frac{(s_1^2/n_1 + s_2^2/n_2)^2}{[(s_1^2/n_1)^2/(n_1 + 1)] + [(s_2^2/n_2)^2/(n_2 + 1)]} \right\} - 2 \tag{1.21}$$

where $\overline{x}_1$ and $\overline{x}_2$ are the calculated means of the two methods, s_1^2 and s_2^2 are the calculated standard deviations of the two methods, and n_1 and n_2 are the number of measurements in the two methods.

At first sight these four equations may appear daunting, but closer inspection reveals that they are simply based on variance (s^2), mean ($\overline{x}$) and number of analytical measurements (n) and that the mathematical manipulation of the data is relatively easy.

Example 9 COMPARISON OF TWO ANALYTICAL METHODS USING REPLICATED ANALYSIS OF A SINGLE TEST SAMPLE

Question

A sample of fasting serum was used to evaluate the performance of the glucose oxidase and hexokinase methods for the quantification of serum glucose concentrations (for details, see Section 15.2.5). The following replicated values were obtained: for the glucose oxidase method 2.3, 2.5, 2.2, 2.6 and 2. 5 mM and for the hexokinase method 2.1, 2.7, 2.4, 2.4 and 2.2 mM. Establish whether or not the two methods gave the same results at the 95% confidence level.

Answer

Using the standard formulae (equations 1.12 and 1.14) we can calculate the mean, standard deviation and variance for each data set.

Glucose oxidase method:

$\overline{x} = 2.42$ mM, $s = 0.16$ mM, $s^2 = 0.026$ (mM)^2

Hexokinase method:

$\overline{x} = 2.36$ mM, $s = 0.23$ mM, $s^2 = 0.053$ (mM)^2

We can then apply the *F*-test to the two variances to establish whether or not they are the same:

$$F_{calc} = \frac{0.053}{0.026} = 2.04$$

F_{table} for the two sets of data each with 4 degrees of freedom and for the 95% confidence level is 6.39 (Table 1.11).

Since F_{calc} is less than F_{table} we can conclude that the two variances are not significantly different. Therefore, using equations 1.18 and 1.19, we can calculate that:

$$S_{pooled} = \sqrt{\frac{0.16(4) + 0.23(4)}{8}} = \sqrt{\frac{0.64 + 0.92}{8}} = \sqrt{0.195} = 0.442$$

$$t_{calc} = \frac{2.42 - 2.36}{0.442}\sqrt{\frac{(5)(5)}{10}} = (0.06/0.442)(1.58) = 0.21$$

Using Table 1.9 at the 95% confidence level and for 8 degrees of freedom t_{table} is 2.306. Thus t_{calc} is far less than t_{table} and so the two sets of data are not significantly different, i.e. the two methods have given the same result at the 95% confidence level.

Table 1.11 Critical values of *F* at the 95% confidence level

Degrees of freedom for S_2	Degrees of freedom for S_1							
	2	3	4	6	10	15	30	∞
2	19.0	19.2	19.2	19.3	19.4	19.4	19.5	19.5
3	9.55	9.28	9.12	8.94	8.79	8.70	8.62	8.53
4	6.94	6.59	6.39	6.16	5.96	5.86	5.75	5.63
5	5.79	5.41	5.19	4.95	4.74	4.62	4.50	4.36
6	5.14	4.76	4.53	4.28	4.06	3.94	3.81	3.67
7	4.74	4.35	4.12	3.87	3.64	3.51	3.38	3.23
8	4.46	4.07	3.84	3.58	3.35	3.22	3.08	2.93
9	4.26	3.86	3.63	3.37	3.14	3.01	2.86	2.71
10	4.10	3.71	3.48	3.22	2.98	2.84	2.70	2.54
15	3.68	3.29	3.06	2.79	2.54	2.40	2.25	2.07
20	3.49	2.10	2.87	2.60	2.35	2.20	2.04	1.84
30	3.32	2.92	2.69	2.42	2.16	2.01	1.84	1.62
∞	3.00	2.60	2.37	2.10	1.83	1.67	1.46	1.00

Comparison of two competitive analytical methods – paired *t*-test

A variant of the previous type of comparison of two analytical methods, based upon the analysis of a common standard sample, is the case in which a series of test samples is analysed once by the two different analytical methods. In this case there is no replication of analysis of any test sample by either method. The *t*-test is applied to the differences between the results of each method for each test sample. This is an example of a paired *t*-test. The formula for calculating t_{calc} in this case is given by equation 1.22:

$$t_{calc} = \frac{\bar{d}}{s_d}\sqrt{n} \tag{1.22}$$

$$s_d = \sqrt{\frac{\Sigma(d_i - \bar{d})^2}{n-1}} \tag{1.23}$$

where d_i is the difference between the paired results, $\bar{d}$ is the mean difference between the paired results, n is the number of paired results, and s_d is the standard deviation of the differences between the pairs.

Example 10 COMPARISON OF TWO ANALYTICAL METHODS USING DIFFERENT TEST SAMPLES

Question

Ten fasting serum samples were each analysed by the glucose oxidase and hexokinase methods. The following results, in mM, were obtained:

Glucose oxidase (mM)	Hexokinase (mM)	Difference, d_i	Difference minus mean of difference	(Difference minus mean of difference)^2
1.1	0.9	0.2	0.08	0.0064
2.0	2.1	−0.1	−0.22	0.0484
3.2	2.9	0.3	0.18	0.0324
3.7	3.5	0.2	0.08	0.0064
5.1	4.8	0.3	0.18	0.0324
8.6	8.7	−0.1	−0.22	0.0484
10.4	10.6	−0.2	−0.32	0.1024
15.2	14.9	0.3	0.18	0.0324
18.7	18.7	0.0	−0.12	0.0144
25.3	25.0	0.3	0.18	0.0324
		Mean ($\bar{d}$) 0.12		Σ 0.3560

Do the two methods give the same results at the 95% confidence level?

Answer

Before addressing the main question, note that the 10 samples analysed by the two methods were chosen to cover the whole analytical range for the methods. To assess whether or not the two methods have given the same result at the chosen confidence level, it is necessary to calculate a value for t_{calc} and to compare it with t_{table} for the 9 degrees of freedom in the study. To calculate t_{calc}, it is first necessary to calculate the value of s_d in equation 1.23. The appropriate calculations are shown in the table above.

$$s_d = \sqrt{[\Sigma(d_i - \bar{d})^2]/(n-1)}$$

$$= \sqrt{(0.356/9)}$$

$$= 0.199$$

From equation 1.22

$$t_{calc} = \frac{\bar{d}\sqrt{n}}{s_d}$$

$$= (0.12\sqrt{10})/0.199$$

$$= 1.907$$

Using Table 1.9, t_{table} at the 95% confidence level and for 9 degrees of freedom is 2.262. Since t_{calc} is smaller than t_{table} the two methods do give the same results at the 95% confidence level. Inspection of the two data sets shows that the glucose oxidase method gave a slightly high value for 7 of the 10 samples analysed.

An alternative approach to the comparison of the two methods is to plot the two data sets as an x/y plot and to carry out a regression analysis of the data. If this is done using the glucose oxidase data as the y variable, the following results are obtained:

Slope: 1.0016, intercept: 0.1057, correlation coefficient r: 0.9997

The slope of very nearly 1 confirms the similarity of the two data sets, whilst the small positive intercept on the y-axis confirms that the glucose oxidase method gives a slightly higher, but insignificantly different, value from that of the hexokinase method.

1.6.6 Calibration methods

Quantitative biochemical analyses often involve the use of a calibration curve produced by the use of known amounts of the analyte using the selected analytical procedure. A calibration curve is a record of the measurement (absorbance, peak area, etc.) produced by the analytical procedure in response to a range of known quantities of the standard analyte. It involves the preparation of a standard solution of the analyte and the use of a range of aliquots in the test analytical procedure. It is good practice to replicate each calibration point and to use the mean $\pm$ 1 SD for the construction of the calibration plot. Inspection of the compiled data usually reveals a scatter of the points about a linear relationship but such that there are several options for the 'best' fit. The technique of fitting the best fit 'by eye' is not recommended, as it is highly subjective and irreproducible. The method of least mean squares linear regression (LMSLR) is the most common mathematical way of fitting a straight line to data but, in applying the method, it is important to realise that the accuracy of the values for slope and intercept that it gives are determined by experimental error built into the x and y values.

The mathematical basis of LMSLR is complex and will not be considered here, but the principles upon which it is based are simple. If the relationship between the two variables, such as the concentration or amount of analyte and response, is linear, then the 'best' straight line will have the general form $y = mx + c$, where x and y are the two variables, m is the slope of the line and c is the intercept on the y-axis. It is assumed first, correctly in most cases, that the errors in the measurement of y are much greater than those for x (it does not assume that there are no errors in the x values) and, secondly, that uncertainties (standard deviations) in the y values are all of the same magnitude. The method uses two criteria. The first is that the line will pass through the point $(\overline{x},\overline{y})$ where $\overline{x}$ and $\overline{y}$ are the mean of the x and y values respectively. The second is that the slope (m) is based on the calculation of the optimum values of m and c that give minimum variation between individual experimental y values and their corresponding values as predicted by the 'best' straight line. Since these variations can be positive or negative (i.e. the experimental values can be greater or smaller than those predicted by the 'best' straight line), in the process of arriving at the best slope the method measures the deviations between the experimental and candidate straight line values, squares them (so they are all positive), sums them and then selects the values of m and c that give the minimum deviations. The end result of the regression analysis is the equation for the best-fit straight line for the experimental data set. This is then used to construct the calibration curve and subsequently to analyse the test analyte(s). Most modern calculators will carry out this type of analysis and will simultaneously report the 95% confidence limits for the m and c values and/or the standard deviation associated with the two values together with the 'goodness-of-fit' of the data as expressed by a correlation coefficient r or a coefficient of determination r^2. The stronger the correlation between the two variables, the closer the value of r approaches $+1$ or -1. Values of r are quoted to four decimal places and for good

correlations commonly exceed 0.99. Values of 0.98 and less should be considered with care, since even slight curvature can give r values of this order.

In the routine construction of a calibration curve, a number of points have to be borne in mind:

- *Selection of standard values:* A range of standard analyte amounts/concentrations should be selected to cover the expected values for the test analyte(s) in such a way that the values are equally distributed along the calibration curve. Test samples should not be estimated outside this selected range, as there is no evidence that the regression analysis relationship holds outside the range. It is good practice to establish the analytical range and the limit of detection for the method. It is also advisable to determine the precision (standard deviation) of the method at different points across the analytical range and to present the values on the calibration curve. Such a plot is referred to as a precision profile. It is common for the precision to decrease (standard deviation to increase) at the two ends of the curve and this may have implications for the routine use of the curve. For example, the determination of testosterone in male and female serum requires the use of different methods, since the two values (reference range 10–30 nM for males, <3 nM for females) cannot be accommodated with acceptable precision on one calibration curve.
- *Use of a 'blank' sample:* This is where no standard analyte is present. One should be included in the experimental design when possible (it will not be possible, for example, with analyses based on serum or plasma). Any experimental value, for example absorbance, obtained for it must be deducted from all other measurements. This may be achieved automatically in spectrophotometric measurements by the use of a double-beam spectrophotometer in which the blank sample is placed in the reference cell.
- *Shape of curve:* It should not be assumed that all calibration curves are linear. They may be curved, and best represented by a quadratic equation of the type $y = ax^2 + bx + c$ where a, b and c are constants, or they may be logarithmic.
- *Recalibration:* A new calibration curve should be constructed on a regular basis. It is not acceptable to rely on a calibration curve produced on a much earlier occasion.

1.6.7 Internal standards

An additional approach to the control of time-related minor changes in a calibration curve and the quantification of an analyte in a test sample is the use of an internal standard. An ideal internal standard is a compound that has a molecular structure and physical properties as similar as possible to those of the test analyte and which gives a response to the analytical method similar to that of the test analyte. This response, expressed on a unit quantity basis, may be different from that for the test analyte but, provided that the relative response of the two compounds is constant, the advantages of the use of the internal standard are not

compromised. Quite commonly the internal standard is a structural or geometrical isomer of the test analyte.

A known fixed quantity of the standard is added to each test sample and analysed alongside the test analyte by the standard analytical procedure. The resulting response for the standard and the range of amounts or concentrations of the test analyte is used to calculate a relative response for the test analyte and used in the construction of the calibration curve. The curve therefore consists of a plot of the relative response to the test analyte against the range of quantities of the analyte.

Internal standards are commonly used in liquid and gas–liquid chromatography, since they help to compensate for small temporal variations in the flow of liquid or gas through the chromatographic column. In such applications it is, of course, essential that the internal standard runs near to, but distinct from, the test analyte on the chromatograph.

If the analytical procedure involves preliminary sampling procedures, such as solid-phase extraction, it is important that a known amount of the internal standard is introduced into the test sample at as early a stage as possible and is therefore taken through the preliminary procedures. This ensures that any loss of the test analyte during these preliminary stages will be compensated for by identical losses to the internal standard, so that the final relative response of the method to the two compounds is a true reflection of the quantity of the test analyte.

1.7 PRINCIPLES OF CLINICAL BIOCHEMICAL ANALYSIS

1.7.1 Basis of analysis of body fluids for diagnostic, prognostic and monitoring purposes

Many human diseases are either the result of abnormal metabolism or the cause of a perturbation of normal cellular activity. In both cases there is a characteristic and significant change in the biochemical profile of body fluids. The application of quantitative analytical biochemical tests to a large range of biological analytes in body fluids and tissues is a valuable aid to the diagnosis and management of the prevailing disease state. In this section the general biological and analytical principles underlying these tests will be discussed and related to the general principles of quantitative chemical analysis discussed in Section 1.6.

Body fluids such as blood, cerebrospinal fluid and urine in both healthy and diseased states contain a large number of inorganic ions and organic molecules. Whilst the normal biological function of some of these chemical species lies within that fluid, for the majority it does not. The presence of this latter group of chemical species within the fluid is due to the fact that normal cellular secretory mechanisms, and the temporal synthesis and turnover of individual cells and their organelles within the major organs of the body, all result in the release of cell components, particularly those located in the cytoplasm, into the surrounding extracellular fluid and eventually into the blood circulatory system. This in turn transports them to the main excretory organs, namely the liver, kidneys and lungs, so that these cell components and/or their degradation products are eventually

excreted in faeces, urine and expired air. Examples of cell components in this category include enzymes, hormones, intermediary metabolites and small organic and inorganic ions.

The concentration, amount or activity of a given cell component that can be detected in these fluids of a healthy individual at any point in time depends on many factors that can be classified into one of three categories, namely chemical characteristics of the component, endogenous factors characteristic of the individual, and exogenous factors that are imposed on the individual.

- *Chemical characteristics:* Some molecules are inherently unstable outside their normal cellular environment. For example, some enzymes are reliant on the presence of their substrate and/or coenzyme for their stability and these may be absent or in too low concentrations in the extracellular fluid. Molecules that can act as substrates of catabolic enzymes found in extracellular fluids, in particular blood, will also be quickly metabolised. Cell components that fall into these two categories therefore have a short half-life outside the cell and are normally present in low concentrations in fluids such as blood.
- *Endogenous factors:* These include age, gender, body mass and pregnancy. For example:

  (a) serum cholesterol concentrations are higher in men than in pre-menopausal women, but the differences decreases post-menopause;
  (b) serum alkaline phosphatase activity is higher in children than in adults and is raised in women during pregnancy;
  (c) serum insulin and triglyceride concentrations are higher in obese individuals than in the lean;
  (d) serum creatinine, a metabolic product of creatine important in muscle metabolism, is higher in individuals with a large muscle mass;
  (e) serum sex hormone concentrations differ between males and females and change with age.

- *Exogenous factors:* These include time, exercise, food intake and stress. Several hormones are secreted in a time-related fashion. Thus cortisol and to a lesser extent thyroid-stimulating hormone (TSH) and prolactin all show a diurnal rhythm in their secretion. In the case of cortisol, its secretion peaks around 9:00 a.m. and declines during the day, reaching a trough between 11:00 p.m. and 5:00 a.m. The secretion of female sex hormones varies during the menstrual cycle, and that of 25-hydroxycholecalciferol (vitamin D_3) varies with the seasons, peaking during the late summer months. The concentrations of glucose, triglycerides and insulin in blood rise shortly after the intake of a meal. Stress, including that imposed by the process of taking a blood sample by puncturing a vein (venipuncture), can stimulate the secretion of a number of hormones and neurotransmitters including prolactin, cortisol, adrenocorticotrophic hormone (ACTH) and adrenaline.

The influence of these various factors on the extent of release of cell components into extracellular fluids inevitably means that, even in healthy individuals,

there is a considerable intra-individual variation (i.e. variation from one occasion to another in one individual) in the value of any chosen test analyte of diagnostic importance and an even larger inter-individual variation (i.e. variation between individuals). More importantly, the superimposition of a disease state onto these causes of intra- and inter-individual variation will result in an even greater variability between test occasions.

Many clinical conditions compromise the integrity of cells located in the organs affected by the condition. This may result in the cells becoming more 'leaky' or, in more severe cases, actually dying (necrosis) and releasing their contents into the surrounding extracellular fluid. In the vast majority of cases the extent of release of specific cell components into the extracellular fluid, relative to the healthy reference range, will reflect the extent of organ damage and this relationship forms the basis of diagnostic clinical biochemistry. If the cause of the organ damage continues for a prolonged time and is essentially irreversible (i.e. the organ does not undergo self-repair), as is the case in cirrhosis of the liver for example, then the mass of cells remaining to undergo necrosis will progressively decline so that eventually the release of cell components into the surrounding extracellular fluid will decrease even though organ cells are continuing to be damaged. In such cases the measured amounts will not reflect the extent of organ damage.

Clinical biochemical tests have been developed to complement in four main ways a provisional clinical diagnosis based on the patient's medical history and clinical examination:

- *To support or reject a provisional diagnosis* by detecting and quantifying abnormal amounts of test analytes consistent with the diagnosis. For example, serum myoglobin, troponin I (part of the cardiac contractile muscle), creatine kinase (specifically the CK-MB isoform; Section 1.7.3) and aspartate transaminase all rise following a myocardial infarction (heart attack), which results in cell death in some heart tissue. Tests can also help a differential diagnosis, for example in distinguishing the various forms of jaundice (yellowing of the skin owing to the presence of the yellow pigment bilirubin, a metabolite of haem) by the measurement of alanine transaminase and aspartate transaminase activities and by determining whether or not the bilirubin is conjugated with β-glucuronic acid.
- *To monitor recovery following treatment* by repeating the tests on a regular basis and monitoring the return of the test values to those within the reference range. Following a myocardial infarction, for example, the raised serum enzyme activities referred to above usually return to reference range values within 10 days (Section 1.7.3, see Fig. 1.6). Similarly, the measurement of serum tumour markers such as CA125 can be used to follow recovery or recurrence after treatment for ovarian cancer.
- *To screen for latent disease* in apparently healthy individuals by testing for raised levels of key analytes; for example, measuring serum glucose for diabetes mellitus and immunoreactive trypsin for cystic fibrosis. It is now common for serum cholesterol levels to be used as a measure of the risk of an individual

developing heart disease. This is particularly important for individuals with a family history of the disease. An action limit of serum cholesterol <5.2 mM has been set by the British Hyperlipidaemia Association for an individual to be counselled on the importance of a healthy (low fat) diet and regular exercise and a higher action limit of serum cholesterol >6.6 mM for cholesterol-lowering 'statin' drugs to be prescribed and clinical advice given.

- *To detect toxic side-effects of treatment*, for example in patients receiving hepatotoxic drugs, by undertaking regular liver function tests. An extension of this is therapeutic drug monitoring, in which patients receiving drugs such as phenytoin and carbamezepine (both of which are used in the treatment of epilepsy) that have a low therapeutic index (ratio of the dose required to produce a toxic effect relative to the dose required to produce a therapeutic effect) are regularly monitored for drug levels and liver function to ensure that they are receiving effective and safe therapy.

Reference ranges

For a biochemical test for a specific analyte to be routinely used as an aid to clinical diagnosis, it is essential that the test has the required performance indicators (Section 1.6.2) especially specificity and sensitivity. Sensitivity expresses the proportion of patients with the disease who are correctly identified by the test. Specificity expresses the proportion of patients without the disease who are correctly identified by the test. These two parameters may be expressed mathematically as follows:

$$\text{sensitivity} = \frac{\text{true positive tests} \times 100\%}{\text{total patients with the disease}}$$

$$\text{specificity} = \frac{\text{true negative tests} \times 100\%}{\text{total patients without the disease}}$$

Ideally both of these indicators for a particular test should be 100% but this is not always the case. This problem is most likely to occur in cases where the change in the amount of the test analyte in the clinical sample is small as compared with the reference range values found in healthy individuals. Both of these indicators express the performance of the test but it is equally important to be able to quantify the probability that the patient with a positive test has the disease in question. This is best achieved by the predictive power of the test. This expresses the proportion of patients with a positive test who are correctly diagnosed as disease positive:

$$\text{positive predictive value} = \frac{\text{true positive patients}}{\text{total positive tests}}$$

$$\text{negative predictive value} = \frac{\text{true negative patients}}{\text{total negative tests}}$$

The concept of predictive power can be illustrated by reference to fetal screening for Down's syndrome and neural tube defects. Preliminary tests for these

conditions in unborn children are based on the measurement of α-fetoprotein (AFP), human chorionic gonadotrophin (hCG) and unconjugated oestriol (uE_3) in the mother's blood. The presence of these conditions results in an increased hCG and decreased AFP and uE_3 relative to the average in healthy pregnancies. The results of the tests are used in conjunction with the gestational and maternal ages to calculate the risk of the baby suffering from these conditions. If the risk is high, further tests are undertaken, including the recovery of some fetal cells for genetic screening from the amniotic fluid surrounding the fetus in the womb by inserting a hollow needle into the womb (amniocentesis). The three tests detect two out of three cases (67%) of Down's syndrome and four out of five cases (80%) of neural tube defects. Thus the performance indicators of the tests are not 100% but they are sufficiently high to justify their routine use.

The correct interpretation of all biochemical test data is heavily dependent upon the use of the correct reference range against which the test data are to be judged. As pointed out above, the majority of biological analytes of diagnostic importance are subject to considerable inter- and intra-individual variation in healthy adults, and the analytical method chosen for a particular analyte assay will have its own precision, accuracy and selectivity that will influence the analytical results. In view of these biological and analytical factors, individual laboratories must establish their own reference range for each test analyte, using their chosen methodology and a large number (hundreds) of 'healthy' individuals. The recruitment of individuals to be included in reference range studies presents a considerable practical and ethical problem owing to the difficulty of defining 'normal' and of using invasive procedures, such as venipuncture, to obtain the necessary biological samples. The establishment of reference ranges for children, especially neonates, is a particular problem.

Reference ranges are most commonly expressed as the range that covers the mean ± 1.96 standard deviations of the mean of the experimental population. This range covers 95% of the population. The majority of reference ranges are based on a normal distribution of individual values but, in some cases, the experimental data is asymmetric, often being skewed to the upper limits. In such cases it is normal to use logarithmic data to establish the reference range but, even so, the range may overlap with values found in patients with the test disease state. Typical reference ranges are shown in Table 1.12.

1.7.2 The operation of clinical biochemistry laboratories

The clinical biochemistry laboratory in a typical general hospital in the UK serves a population of about 400 000, containing approximately 60 general practitioner (GP) groups, depending upon the location in the UK. This population will generate approximately 1200 requests from GPs and hospital doctors each weekday for clinical biochemical tests on their patients. Each patient request will require the laboratory to undertake an average of seven specific analyte tests. The result is that a typical general hospital laboratory will carry out between 2.5 and 3 million tests each year. The majority of clinical biochemistry laboratories offers the local

Table 1.12 Typical reference ranges for biochemical analytes

Analyte	Reference range	Comment
Sodium	133–145 mM	
Potassium	3.5–5.0 mM	Values increased by haemolysis or prolonged contact with cells
Urea	3.5–6.5 mM	Range varies with sex and age, e.g. values up to 12.1 will be found in males over the age of 70
Creatinine	75–115 mM (males) 58–93 mM (females)	Creatinine (a metabolite of creatine) production relates to muscle mass and is also a reflection of renal function. Values for both sexes increase by 5–20% in the elderly
Aspartate transaminase (AST)	<40 IU dm^{-3}	Perinatal levels are <80 IU dm^{-3} and fall to adult values by the age of 18 years. Some slightly increased values up to 60 IU dm^{-3} may be found in females over the age of 50 years. Results are increased by haemolysis
Alanine transaminase (ALT)	<40 IU dm^{-3}	Higher values are found in males up to the age of 60 years
Alkaline phosphatase (AP)	<122 IU dm^{-3} (adults) <455 IU dm^{-3} (children <12 yr)	Significantly raised results of up to 2- or 3-fold would be experienced during growth spurts through teenage years. Slightly raised levels also seen in the elderly and in women during pregnancy
Cholesterol	No reference range but recommended value of <5.2 mM	The measurement of cholesterol in an adult 'well' population does not show a Gaussian distribution but a very tailed distribution with relatively few low results. The majority are <10 mM but there is a long tail up to over 20 mM. There is a tendency for males to have a higher cholesterol than females of the same age but, after the menopause, female values revert to those of males. Generally values increase with age

medical community as many as 200 different clinical biochemical tests that can be divided into eight categories as shown in Table 1.13.

Most of the requests for biochemical tests will arise on a routine daily basis but some will arise from emergency medical situations at any time of the day. The large number of daily test samples coupled with the need for a 24 hour, 7 days per week service dictates that the laboratory must rely heavily on automated analysis to carry out the tests and on information technology to process the data.

To achieve an effective service, a clinical biochemistry laboratory has three main functions:

- to advise the requesting GP or hospital doctor on the appropriate tests for a particular medical condition and on the collection, storage and transport of the patient samples for analysis;

Table 1.13 Examples of biochemical analytes used to support clinical diagnosis

Type of analyte	Examples
Food stuffs entering the body	Cholesterol, glucose, fatty acids, triglycerides
Waste products	Bilirubin, creatinine, urea
Tissue-specific messengers	Adrenocorticotrophic hormone (ACTH), follicle-stimulating hormone (FSH), lutenising hormone (LH), thyroid-stimulating hormone (TSH)
General messengers	Cortisol, insulin, thyroxine
Response to messengers	Glucose tolerance test assessing the appropriate secretion of insulin. Tests of pituitary function
Organ function	Adrenal function – cortisol, ACTH Renal function – K^+, Na^+, urea, creatinine Thyroid function – free thyroxine (FT_4), free tri-iodothyronine (FT_3), TSH
Organ disease markers	Heart – troponin I, creatine kinase (CK-MB), AST, lactate dehydrogenase (LD) Liver – ALT, AP, γ-glutamyl transferase (GGT), bilirubin, albumin
Disease specific markers	Specific proteins ('tumour markers') secreted from specific organs – prostate-specific antigen (PSA), CA-125 (ovary), calcitonin (thyroid), α_1-fetoprotein (liver)

- to provide a quality analytical service for the measurement of biological analytes in an appropriate and timely way;
- to provide the requesting doctor with a data interpretation and advice service on the outcome of the biochemical tests and possible further tests.

The advice given to the clinician is generally supported by a User Handbook, prepared by senior laboratory personnel, which includes a description of each test offered, instructions on sample collection and storage, normal laboratory working hours and the approximate time it will take the laboratory to undertake each test. This turnaround time will vary from less than 1 h to several weeks, depending upon the speciality of the test. The vast majority of biochemical tests are carried out on serum or plasma derived from a blood sample. Serum is the preferred matrix for biochemical tests but the concentrations of most test analytes are almost the same in the two fluids. Serum is obtained by allowing the blood to clot and removing the clot by centrifugation. To obtain plasma it is necessary to add an anticoagulant to the blood sample and removing red cells by centrifugation. The two most common anticoagulants are heparin and EDTA, the choice depending on the particular biochemical test required. For example, EDTA complexes calcium ions so that calcium in EDTA plasma would be undetectable. For the measurement of glucose, fluoride/oxalate is added to the sample not as an anticoagulant but to inhibit glycolysis during the transport and storage of the sample. Special vacuum collection tubes containing specific anticoagulants or other additives are available for the storage of blood samples. Collection tubes are also

available containing clot enhancers to speed the clotting process for serum preparation. Many containers incorporate a gel with a specific gravity designed to float the gel between cells and serum providing a barrier between the two for up to 4 days. Biochemical tests may also be carried out on whole blood, urine, cerebrospinal fluid (the fluid surrounding the spinal cord and brain), faeces, sweat, saliva and amniotic fluid. It is essential that the samples are collected in the appropriate container at the correct time (particularly important if the test is for the measurement of hormones such as cortisol subject to diurnal release) and labelled with appropriate patient and biohazard details. Samples submitted to the laboratory for biochemical tests are accompanied by a request form, signed by the requesting clinician, that gives details of the tests required and brief details of the reasons for the request to aid data interpretation and to help identify other appropriate tests.

Laboratory reception

On receipt in the laboratory both the sample and the request form will be assigned an acquisition number, usually in an optically readable form but with a bar code. A check is made of the validity of the sample details on both the request form and sample container to ensure that the correct container for the tests required has been used. Samples may be rejected at this stage if details are not in accordance with the set protocol. Correct samples are then split from the request form and prepared for analysis typically by centrifugation to prepare serum or plasma. The request form is processed into the computer system that identifies the patient against the sample acquisition number, and the tests requested by the clinician typed into the database. It is vital at this reception phase that the sample and patient data match and that the correct details are placed in the database. These details must be adequate to uniquely identify the patient, bearing in mind the number of potential patients in the catchment area, and will include name, address, date of birth, hospital or Accident & Emergency number and acquisition number.

Analytical organisation

The analytical organisation of the majority of clinical biochemical laboratories is based on three work areas:

- autoanalyser section,
- immunoassay section,
- manual section.

Autoanalyser section

Autoanalysers dedicated to clinical biochemical analysis are available from many commercial manufacturers. A typical analyser layout is shown in Fig. 1.5. Analysers have carousels for holding the test samples in racks each carrying up to 15 samples, one or two carousels each for up to 50 different reagents, which are identified by a unique bar code, carousels for sample washing/preparation and a

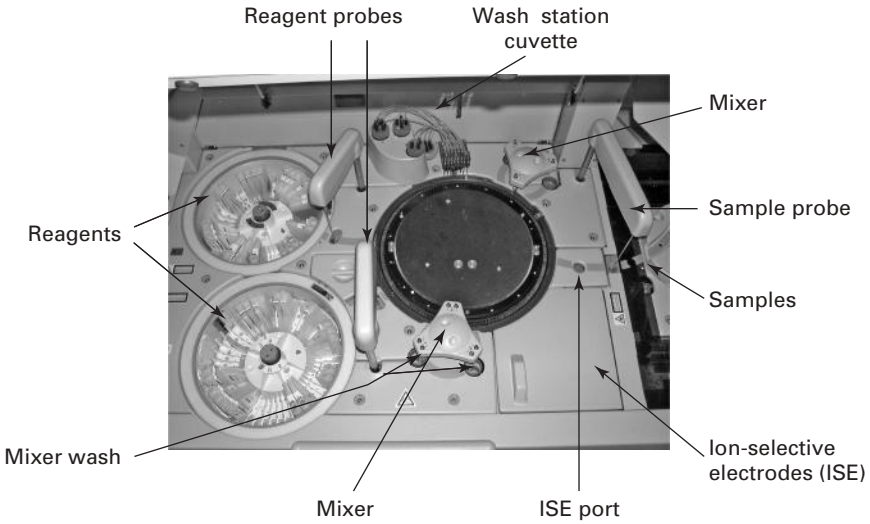

Fig. 1.5. The Olympus 640 autoanalyser (Reproduced by permission of Olympus Diagnostic Division, UK.)

reaction carousel containing up to 200 cuvettes for initiating and monitoring individual test reactions. The analysers have a high throughput capacity with a time cycle that allows additions or readings to be taken in a given cuvette every 4–20 s depending on the analyser model. Multiple analyte test reactions will be taking place and being monitored at any given time. The total reaction time for a given analyte sample will depend on the specific reaction being monitored, but a typical cycle is that for the analysis of serum glucose based on the hexokinase and glucose-6-phosphate dehydrogenase reactions and the monitoring of the increase in absorption at 340 nm due to NADH (Section 15.2.5):

- *Time* 0*:* Add reagent 1 (ATP, Mg^{2+} and hexokinase in Pipes buffer, pH 7.6) to reaction cuvette. Dilute with deionised water and mix.
- *Time* 30 *s:* Add serum test sample. Dilute with deionised water and mix. Monitor absorption at 340 nm for 3 min to obtain sample blank reading.
- *Time* 3 *min* 30 *s:* Add reagent 2 (glucose-6-phosphate dehydrogenase, NAD^+). Mix. Total reaction volume 250 mm^3. Monitor absorption at 340 nm every 20 s.
- *Time* 8 *min:* Record final absorption change due to NADH, allowing for sample blank. Calculate glucose concentration in test sample and record on database. Rinse cuvette. Cycle complete. If the absorption change is outside the calibration range, the analyser will automatically initiate a repeat analysis using a smaller test sample.

The analysers operate with a state-of-the-art spectrophotometer with fibre optics that has multiple read-centres around the reaction carousel that can take readings at several wavelengths simultaneously. Measurements can also be taken with an oxygen electrode and three or four different ion-selective electrodes. The reaction cuvettes may be disposable and automatically loaded and unloaded onto

Table 1.14 Examples of analytical techniques used to quantify analytes by autoanalysers

Analytical technique	Examples of analytes
Ion-selective electrodes	K^+, Na^+, Li^+, Cl^-
Visible and UV spectrophotometry	Urea, creatinine, calcium, urate
Turbidimetric	IgG, IgA, IgM, D-dimer (a metabolic product of fibrinogen)
Reaction rate	Enzymes – AST, ALT, GGT, AP, CK, LD
EMIT	Therapeutic drug monitoring – phenytoin, carbamezapine

UV, ultraviolet; EMIT, enzyme multiplier immunoassay test; AST, aspartate transaminase; ALT, alanine aminotransaminase; GGT, γ-glutamyl transferase; AP, alkaline phosphatase; CK, creatine kinase; LD, lactate dehydrogenase.

the carousel or may be reusable, in which case they are laundered by a sophisticated wash station that uses cycles of acid, alkali and alcohol in various combinations followed by a water wash and an air dry. Disposable cuvettes are collected in sealable containers for disposal and all liquid waste is pumped into containers containing powerful disinfectants. Internal computers as well as an external interface with the main laboratory computer and database control the operation of the analyser. Most analysers have about 25 pre-programmed methods of analysis for a range of analytes, based on five main analytical modes (Table 1.14), but also allow 'in-house' methods to be programmed by laboratory personnel.

Each laboratory will have at least two analysers each offering a similar analytical repertoire so that one can back up the other. The analyser reads the bar code acquisition number for each sample and, on the basis of the reading, interrogates the host computer database to identify the tests to be carried out on the sample. The identified tests are then automatically prioritorised into the most efficient order and the analyser programmed to take the appropriate volume of sample by means of a sampler that may also be capable of detecting microclots in the sample, add the appropriate volume of reagents in a specified order and to monitor the progress of the reaction. Internal quality control samples are also analysed on an identical basis at regular intervals. The analyser automatically monitors the use of all reagents so that it can identify when each will need replenishing. When the test results are calculated, the operator can validate them either on the analyser or on the main computer database. When it is appropriate, the results can also be checked against previous results on the same patient.

Immunoassay section

Immunoassay procedures undertaken by modern autoanalysers are based mostly on fluorescence or polarised fluorescence techniques. The range of analytes varies from manufacturer to manufacturer but usually involves basic endocrinology (e.g. thyroid fuction tests), therapeutic drugs (theophylline, digoxin) and drugs of abuse (opiates, cannabis). The operation of autoanalysers in immunoassay mode

is similar to that described above and the results are generally reported on the same day, and are compared with the previous set of results for the patient.

Manual assays section

This approach to biochemical tests is generally more labour intensive than the other two sections and covers a range of analytical techniques such as acetate or gel electrophoresis, immunoelectrophoresis and some more difficult basic spectrophotometric assays. Examples include the assays for catecholamines (for the diagnosis of phaeochromocytoma), 5-hydroxyindole acetic acid (for the diagnosis of carcinoid syndrome) or haemoglobinA_{1c} (for the monitoring of diabetes).

Result reporting

The instrument operator or the section leader initially validates analytical results. This validation process will, in part, be based on the use of internal quality control procedures for individual analytes. Quality control samples are analysed at least twice daily or are included in each batch of test analytes. The analytical results are then subject to an automatic process that identifies results that are either significantly abnormal or require clinical comment or interpretation against rules set by senior laboratory staff.

1.7.3 Diagnostic enzymology

The measurement of the activities or masses of selected enzymes in serum is a long established aid to clinical diagnosis and prognosis. The enzymes found in serum can be divided into three categories based on the location of their normal physiological function:

- *Serum-specific enzymes:* The normal physiological function of these enzymes is based in serum. Examples include the enzymes associated with lipoprotein metabolism and with the coagulation of blood.
- *Secreted enzymes:* These are closely related to the serum-specific enzymes. Examples include pancreatic lipase, prostatic acid phosphatase and salivary amylase.
- *Non-serum-specific enzymes:* These enzymes have no physiological role in serum. They are released into the extracellular fluid and, as a consequence, appear in serum as result of normal cell turnover or, more abundantly, as a result of cell membrane damage, cell death or morphological changes to cells such as those in cases of malignancy. Their normal substrates and/or cofactors may be absent or in low concentrations in serum.

Serum enzymes in the third category are of the greatest diagnostic value. When a cell is damaged, the contents of the cell are released over a period of several hours, with enzymes of the cytoplasm appearing first, since their release is dependent only on the impairment of the integrity of the plasma membrane. The release of these enzymes following cell membrane damage is facilitated by their large concentration gradient, in excess of 1000-fold, across the membrane. The

integrity of the cell membrane is particularly sensitive to events that impair energy production, for example by the restriction of oxygen supply. It is also sensitive to toxic chemicals including some drugs, microorganisms, certain immunological conditions and genetic defects. Enzymes released from cells by such events may not necessarily be found in serum in the same relative amounts as were originally present in the cell. Such variations reflect differences in the rate of their metabolism and excretion from the body and hence of differences in their serum half-lives. This may be as short as a few hours (intestinal alkaline phosphatase, glutathione *S*-transferase, creatine kinase) or as long as several days (liver alkaline phosphatase, alanine aminotransferase, lactate dehydrogenase).

The clinical exploitation of non-serum-specific enzyme activities is influenced by several factors:

- *Organ specificity:* Few enzymes are unique to one particular organ but fortunately some enzymes are present in much larger amounts in some tissues than in others. As a consequence, the relative proportions (pattern) of a number of enzymes found in serum are often characteristic of the organ of origin.
- *Isoenzymes:* Some clinically important enzymes exist in isoenzyme forms and in many cases the relative proportion of the isoenzymes varies considerably between tissues so that measurement of the serum isoenzymes allows their organ of origin to be deduced.
- *Reference ranges:* The activities of enzymes present in the serum of healthy individuals are invariably smaller than that in the serum of individuals with a diagnosed clinical condition such as liver disease. In many cases, the extent to which the activity of a particular enzyme is raised by the disease state is a direct indicator of the extent of cellular damage to the organ of origin.
- *Variable rate of increase in serum activity:* The rate of increase in the activity of released enzymes in serum following cell damage in a particular organ is a characteristic of each enzyme. Moreover, the rate at which the activity of each enzyme decreases towards the reference range following the event that caused cell damage and the subsequent treatment of the patient is a valuable indicator of the patient's recovery from the condition.

The practical implication of these various points to the applications of diagnostic enzymology is illustrated by its use in the management of heart disease and liver disease.

Ischaemic heart disease and myocardial infarction

The healthy functioning of the heart is dependent upon the availability of oxygen. This oxygen availability may be compromised by the slow deposition of cholesterol-rich atheromatous plaques in the coronary arteries. As these deposits increase, a point is reached at which the oxygen supply cannot be met at times of peak demand, for example at times of strenuous exercise. As a consequence, the heart becomes temporarily ischaemic ('lacking in oxygen') and the individual experiences severe chest pain, a condition known as angina pectoris ('angina of

effort'). Although the pain may be severe during such events, the cardiac cells temporarily deprived of oxygen are not damaged and do not release their cellular contents. However, if the arteries become completely blocked either by the plaque or by a small thrombus (clot) that is prevented from flowing through the artery by the plaque, the patient experiences a myocardial infarction ('heart attack', MI) characterised by the same severe chest pain, but in this case the pain is accompanied by the irreversible damage to the cardiac cells and the release of their cellular contents. This release is not immediate, but occurs over a period of many hours. From the point of view of the clinical management of the patient, it is important for the clinician to establish whether or not the chest pain was accompanied by a MI. In about one-fifth of the cases of a MI event the patient does not experience the characteristic chest pain ('silent MI') but again it is important for the clinician to be aware that the event has occurred. Electrocardiogram (ECG) patterns are a primary indicator of these events but in atypical presentations ECG changes may be ambiguous and additional evidence is sought in the form of changes in serum enzyme activities. The activities of three enzymes are commonly measured:

- *Creatine kinase (CK):* This enzyme converts phosphocreatine (important in muscle metabolism) to creatine. CK is a dimeric protein composed of two monomers, one denoted as M (muscle) the other as B (brain), so that three isoforms exist, CK-MM, CK-MB and CK-BB. The tissue distribution of these isoenzymes is significantly different such that heart muscle consists of 80–85% MM and 15–20% MB, skeletal muscle 99% MM and 1% MB and brain, stomach, intestine and bladder predominantly BB. CK activity is raised in a number of clinical conditions but since the CK-MB form is almost unique to the heart, its raised activity in serum gives unambiguous support for a MI even in cases in which the total CK activity remains within the reference range. A rise in total serum CK activity is detectable within 6 h of the MI and the serum activity reaches a peak after 24–36 h. However, a rise in CK-MB is detectable within 3–4 h, has 100% sensitivity within 8–12 h and reaches a peak within 10–24 h. It remains raised for 2–4 days.
- *Aspartate aminotransferase (AST):* This is one of a number of transaminases involved in intermediary metabolism. It is found in most tissues but is abundant in heart and liver. Its activity in serum is raised following a MI and reaches a peak between 48 and 60 h. It has little clinical value in the early diagnosis of heart muscle damage but is of use in the case of delayed presentation with chest pain.
- *Lactate dehydrogenase (LD):* This is a tetrameric protein made of two monomers denoted as H (heart) and M (muscle) so that five isoforms exist: LD-1 (H4), LD-2 (H3M), LD-3 (H2M2), LD-4 (HM3) and LD-5 (M5). LD-1 predominates in heart, brain and kidney and LD-5 in skeletal muscle and liver. Total LD activity and LD-1 activity in serum increases following a MI and reaches a peak after 48–72 h. The subsequent decline in activity is much slower that that of CK or AST. The diagnostic value of LD activity measurement is mainly confined to monitoring the patient's recovery from the MI event.

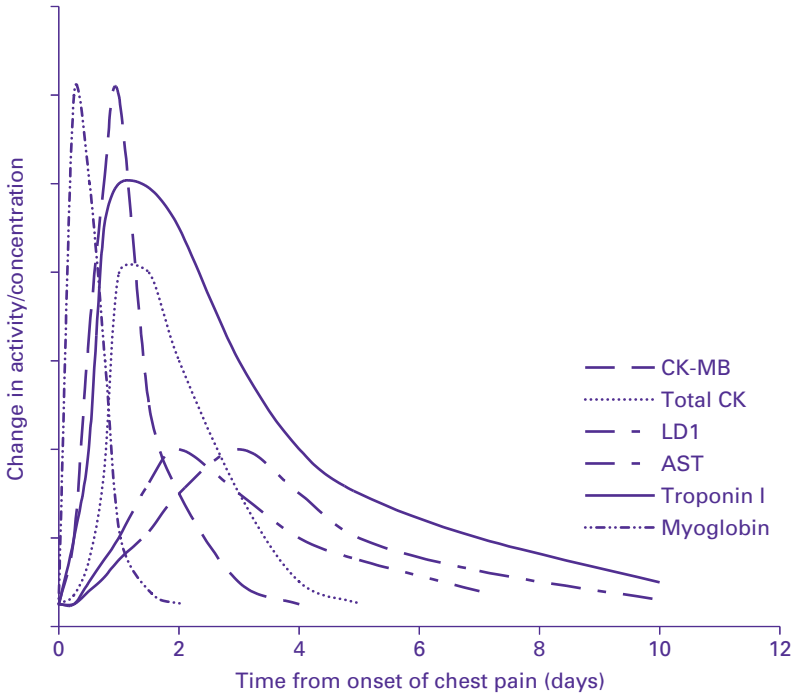

Fig. 1.6. Serum enzyme activity and myoglobin and troponin I concentration changes following a myocardial infarction. Changes are expressed as a multiple of the upper limit of the reference range. Values vary according to the severity of the event, but the time course of each profile is characteristic of all events. CK, creatine kinase; LD, lactate dehydrogenase; AST, aspartate transaminase.

Typical changes in the activities of these three enzymes following a MI are shown in Fig. 1.6. All three enzymes are assayed by an automated method based on the following reactions.

- *Total CK activity:* This is assessed by coupled reactions (Section 15.2.2) with hexokinase and glucose-6-phosphate dehydrogenase in the presence of *N*-acetylcysteine as activator, and the measurement of increase in absorbance at 340 nm or by fluorescence polarisation (primary wavelength 340 nm, reference wavelength 378 nm):

$$\begin{aligned} \text{creatine phosphate} + \text{ADP} &\rightleftharpoons \text{creatine} + \text{ATP} \\ \text{ATP} + \text{D-glucose} &\rightleftharpoons \text{ADP} + \text{D-glucose 6-phosphate} \\ \text{D-glucose 6-phosphate} + \text{NAD(P)}^+ &\rightleftharpoons \text{D-6-phosphogluconate} + \text{NAD(P)H} + \text{H}^+ \end{aligned}$$

- *CK-MB activity:* This is assessed by the inhibition of the activity of the M monomer by the addition to the serum sample of an antibody to the M monomer. This inhibits CK-MM and the M unit of CK-MB. The activity of CK-BB is unaffected but is normally undetectable in serum, hence the remaining activity in serum is due to the B unit of CK-MB. It is assayed by

the above coupled assay procedure and the activity doubled to give an estimate of the CK-MB activity. An alternative assay uses a double antibody technique: CK-MB is bound to anti-CK-MB coated on microparticles, the resulting complex washed to remove non-bound forms of CK and anti-CK-MM conjugated to added alkaline phosphatase. It binds to the antibody–antigen complex, is washed to remove unbound materials and assayed using 4-methylumbelliferone phosphate as substrate, the released 4-methylumbelliferone being measured by its fluorescence and expressed as a concentration (ng cm^{-3}) rather than as activity.

- *Aspartate aminotransferase activity:* This is assessed by a coupled assay with malate dehydrogenase and the measurement of the decrease in absorbance at 340 nm:

  L-aspartate + 2-oxoglutarate $\rightleftharpoons$ oxaloacetate + L-glutamate

  oxaloacetate + NADH + H^+ $\rightleftharpoons$ malate + NAD^+

- *Lactate dehydrogenase:* The measurement of total activity is based on the measurement of the increase in absorbance at 340 nm using lactate as substrate. The measurement of LD-1 is based on the use of 2-hydroxybutyrate as substrate, since only LD-1 and LD-2 can use it:

  Total LD: lactate + NAD^+ $\rightleftharpoons$ pyruvate + NADH + H^+

  LD-1: 2-hydroxybutyrate + NAD^+ $\rightleftharpoons$ 2-ketobutyrate + NADH + H^+

  The clinical importance of obtaining early unambiguous evidence of a myocardial infarction has encouraged the development of markers other than enzyme activities and currently two tests are commonly run alongside enzyme activities. These are based on myoglobin and troponin I:

  Myoglobin: Concentrations of this protein in serum, assayed by HPLC or immunoassay, increase more rapidly than CK-MB after a MI. An increase is detectable within 1–2 h, has 100% sensitivity and reaches a peak within 4–8 h and returns to normal within 12–24 h. However, myoglobin changes are not specific for a MI, since similar changes also occur in other syndromes such as muscle damage or crush injury such as that following a road accident.
  Troponin I: This is one of three proteins (the others being troponin T and troponin C) of a complex that regulate the contractility of the myocardial cells. Its activity in serum increases at the same rate as CK-MB after a myocardial infarction, has a similar time for 100% sensitivity and for peak time, but it remains raised for up to 4 days after the onset of symptoms. Its reference range is less than 1ng cm^{-3} but its concentration in serum is raised to up to 30–50 ng cm^{-3} within 24 h of a MI event. It is assayed by a 'sandwich' immunological assay in which the antibody is labelled with alkaline phosphatase. Using 4-methylumbelliferone phosphate as substrate, the release of 4-methylumbelliferone is measured by fluorescence. The measurement of serum troponin I is widely used to

exclude cardiac damage in patients with chest pain since it remains raised for several days following a MI, but the timing of the test sample is important as a sample taken too early may give a false negative result. A limitation of its use is that its release into serum is not specific to MI, an increase in serum mass may occur following a crush injury.

The measurement of enzyme activities and myoglobin and troponin I concentrations, together with plasma potassium, glucose and arterial blood gases, is routinely used to monitor the recovery of patients following a MI. A patient may experience a second MI within a few days of the first. In such cases the pattern of serum enzymes shown in Fig. 1.6 is repeated, the pattern being superimposed on the remnants of the first profile. CK-MB is the best initial indicator of a second infarction, since the levels of troponin I may not reflect a secondary event.

The sensitivity and specificity (Section 1.7.1) of ECG and diagnostic enzymology in the management of heart disease are complementary. Thus the specificity of ECG is 100% whilst that of enzyme measurements is 90%, and the sensitivity of ECG is 70% whilst that of enzyme measurements is 95%.

Liver disease

Diagnostic enzymology is routinely used to discriminate between several forms of liver disease including:

- *Hepatitis:* General inflammation of the liver most commonly caused by viral infection but which may also be a consequence of blood poisoning (septicaemia) or glandular fever. It results in only mild necrosis of the hepatic cells and hence of a modest release of cellular enzymes.
- *Cirrhosis:* A general destruction of the liver cells and their replacement by fibrous tissue. It is most commonly caused by excess alcohol intake but is also a result of prolonged hepatitis, various autoimmune diseases and genetic conditions. They all result in extensive cell damage and release of hepatic cell enzymes.
- *Malignancy:* Primary and secondary tumours.
- *Cholestasis:* The prevention of bile from reaching the gut due either to blockage of the bile duct by gallstones or tumours or to liver cell destruction as a result of cirrhosis or prolonged hepatitis. This gives rise to obstructive jaundice (presence of bilirubin, a yellow metabolite of haem, in the skin).

Patients with these various liver diseases often present to their doctor with similar symptoms and a differential diagnosis needs to be made on the basis of a range of investigations including imaging techniques especially ultrasonography (ultrasound), magnetic resonance imaging (MRI), computed tomography (CT) scanning, microscopic examination of biopsy samples and liver function tests. Five enzymes are routinely assayed to aid differential diagnosis:

- *Aspartate aminotransferase (AST) and alanine aminotransferase (ALT):* As previously stated, these enzymes are widely distributed but their ratios in serum are characteristic of the specific cause of liver cell damage. For example,

an AST:ALT ratio of less than 1 is found in acute viral hepatitis and fresh obstructive jaundice, a ratio of about 1 in obstructive jaundice caused by viral hepatitis, and a ratio of greater than 1 in cases of cirrhosis.

- *γ-Glutamyl transferase (GGT):* This enzyme transfers a γ-glutamyl group between substrates and may be assayed by the use of γ-glutamyl 4-nitroaniline as substrate and monitoring the release of 4-nitroaniline at 400 nm. GGT is widely distributed and is abundant in liver, especially bile canaliculi, kidney, pancreas and prostate but these do not present themselves by contributing to serum levels. Raised activities are found in cirrhosis, secondary hepatic tumours and cholestasis, and tend to parallel increases in the activity of alkaline phosphatase, especially in cholestasis. Its synthesis is induced by alcohol and some drugs also cause its serum activity to rise.
- *Alkaline phosphatase (AP):* This enzyme is found in most tissues but is especially abundant in the bile canaliculi, kidney, bone and placenta. It may be assayed by using 4-nitrophenylphosphate as substrate and monitoring the release of 4-nitrophenol at 400 nm. Its activity is raised in obstructive jaundice and, when measured in conjunction with ALT, can be used to distinguish between obstructive jaundice and hepatitis, since its activity is raised more than that of ALT in obstructive jaundice. Decreasing serum activity of AP is valuable in confirming an end of cholestasis. Raised serum AP levels can also be present in various bone diseases and during growth and pregnancy.

1.7.4 Quality assessment procedures

In order to validate the analytical precision and accuracy of the biochemical tests conducted by a clinical biochemistry department, the department will participate in external quality assessment schemes in addition to routinely carrying out internal quality control procedures that involve the repeated analysis of reference samples covering the full analytical range for the test analyte. In the UK there are two main national clinical biochemistry external quality assessment schemes: the UK National External Quality Assessment Scheme (UK NEQAS: website <www.ukneqas.org.uk>) coordinated at the Queen Elizabeth Medical Centre, Birmingham and the Wales External Quality Assessment Scheme (WEQAS: website <www.weqas.com>) coordinated at the University Hospital of Wales, Cardiff. The majority of UK hospital clinical biochemistry departments subscribe to both schemes. UK NEQAS and WEQAS distribute test samples on a fortnightly basis, the samples being human serum based. In the case of UK NEQAS the samples contain multiple analytes each at an undeclared concentration within the analytical range. The concentration of each analyte is varied from one distribution to the next. In contrast, WEQAS distributes four or five test samples containing the test analytes at various concentrations within the analytical range. Both UK NEQAS and WEQAS offer a number of quality assessment schemes in which the distributed test samples contain groups of related analytes such as general chemistry analytes, peptide hormones, steroid hormones and therapeutic

drug-monitoring analytes. Participating laboratories elect to subscribe to schemes relevant to their analytical services.

The participating laboratories are required to analyse the external quality assessment samples alongside routine clinical samples and to report the results to the organising centre. Each centre undertakes a full statistical analysis of all the submitted results and reports them back to the individual laboratories on a confidential basis. The statistical data record the individual laboratory's data in comparison with all the submitted data and with the compiled data broken down into individual methods (e.g. the glucose oxidase and hexokinase methods for glucose) and for specific manufacturers' systems. Results are presented in tabular, histogram and graphic form and are compared with the results from recent previously submitted samples. This comparison with previous performance data allows longer-term trends in analytical performance for each analyte to be monitored. Laboratory data that are regarded as unsatisfactory are identified and followed up. Selected data from typical UK NEQAS and WEQAS reports are presented in Table 1.15 and Fig. 1.7.

Clinical audit and accreditation

In addition to participating in external quality assessment schemes, clinical laboratories are also subject to clinical audit. This is a systematic and critical assessment of the general performance of the laboratory against its own declared standards and procedures and against nationally agreed standards. In the context of analytical procedures, the audit evaluates the laboratory performance in terms of: the appropriateness of the use of the tests offered by the laboratory; the clinical interpretation of the results; and the procedures that operate for the receipt, analysis and reporting of the test samples. Thus, whilst it includes the evaluation of analytical data, the audit is concerned primarily with processes leading to the test data with a view to implementing change and improvement. The ultimate objective of the audit is to ensure that the patient receives the best possible care and support in a cost-effective way. The audit is normally undertaken by junior doctors from the hospital, lasts for several days, and involves interaction with all laboratory personnel.

Closely allied to the process of clinical audit is that of accreditation. However, whereas clinical audit is carried out primarily for the local benefit of the laboratory and its staff and ultimately for the patient, accreditation is a public and national recognition of the professional quality and status of the laboratory and its personnel. The accreditation process and assessment is the responsibility of either a recognised public professional body or a government department or agency. Different models operate in different countries. In the UK, accreditation of clinical biochemistry laboratories is voluntary and is carried out by either Clinical Pathology Accreditation (UK) Ltd (CPA) or less commonly the United Kingdom Accreditation Service (UKAS). In the USA, accreditation is mandatory and may be carried out by one of a number of 'deemed authorities' such as the College of American Pathologists. Accreditation organisations also exist for non-clinical analytical laboratories. Examples in the UK include the National Measurement

Table 1.15 Selected UK NEQAS and WEQAS quality assessment data for serum glucose (mM)

(a) **UK NEQAS report** © The data are reproduced by permission of UK NEQAS, Wolfson EQA Laboratory, Birmingham

Analytical method	*n*	Mean	SD	CV (%)
All methods	635	18.68	0.60	3.2
Beckman Glucose Analyser	62	18.22	0.41	2.2
Beckman Synchron CX3/CX7	59	18.25	0.38	2.1
Glucose oxidase/dehydrogenase	148	18.78	0.63	3.4
Roche Hitachi/Modular	75	18.60	0.52	2.8
Other discrete analysers	47	18.89	0.82	4.4
Hexokinase + G6PDH	297	18.84	0.61	3.2
Discrete analyser	47	18.57	0.59	3.2
Olympus systems	132	19.05	0.51	2.7
Roche Integra	51	18.86	0.62	3.3
Ortho Vitros	112	18.46	0.37	2.0
700/750/950	47	18.39	0.40	2.2
250	59	18.52	0.35	1.9

SD, standard deviation; CV, coefficient of variation.
The Beckman Glucose Analyser uses the glucose oxidase method and measures oxygen consumption using an oxygen electrode. The Vitros method is a so-called 'dry chemistry' method that involves placing the sample on a slide, similar to a photographic slide, that has the reagents of the glucose oxidase method impregnated in the emulsion. A blue colour is produced and its intensity measured by reflected light.

These data are for a laboratory that used the hexokinase method and reported a result of 18.7 mM. UK NEQAS calculate a method laboratory trimmed mean (MLTM) as a target value. It is the mean value of all the results returned by all laboratories using the same method with results ±2 SD outside the mean omitted. Its value was 18.84. On the basis of the difference between the MLTM and the laboratory's result, UK NEQAS also calculates a score of the specimen accuracy and bias together with a measurement of the laboratory's consistency of bias. This involves aggregating the bias data from all specimens of that analyte submitted by the laboratory, within the previous 6 months, representing the 12 most recent distributions. This score is an assessment of the tendency of the laboratory to give an over-positive or under-negative estimate of the target MLTM values. The score indicated that the laboratory was consistently underestimating the MLTM but within an acceptable laboratory performance.

The results embodied in this table are shown in histogram form in Fig. 1.7.

Accreditation Service (NAMAS) and the British Standards Institution (BSI). The International Laboratory Accreditation Cooperation (ILAC), the European Co-operation for Accreditation (EA) and the Asia-Pacific Laboratory Accreditation Co-operation (APLAC) are three of many international fora for the harmonisation of national standards of accreditation for analytical laboratories.

Assessors appointed by the accreditation body assess the compliance by the laboratory with standards set by the accreditation body. The standards cover a wide range of issues such as those of accuracy and precision, timeliness of results,

Table 1.15 (*Cont.*)

(b) **WEQAS report** © The data are reproduced by permission of WEQAS, Directorate of Laboratory Medicine, University Hospital of Wales, Cardiff

Analytical method		Sample number 1	2	3	4
Reported result		7.2	3.7	17.2	8.7
Hexokinase	Mean	6.9	3.6	17.0	8.4
	SD	0.2	0.1	0.5	0.3
	Number	220	221	219	219
Aeroset	Mean	7.2	3.8	17.3	8.5
	SD	0.15	0.08	0.045	0.2
	Number	8	8	9	8
Overall	Mean	7.0	3.7	17.2	8.6
	SD	0.28	0.20	0.54	0.33
	Number	388	392	388	389
WEQAS SD		0.26	0.16	0.6	0.3
SDI		1.15	0.63	0.33	1.0

SD, standard deviation; SDI, standard deviation index.
These data are for a laboratory that used the hexokinase method for glucose using an Aeroset instrument. Accordingly, the WEQAS report includes the results submitted by all laboratories using the hexokinase method and all results for the method using an Aeroset. The overall results refer to all methods irrespective of instrument. All the data are 'trimmed' in that results outside ±2 SD of the mean are rejected, which explains why the total number for each test sample varies slightly. WEQAS SD is calculated from the precision profiles for each analyte and the SDI is equal to (the laboratory result minus method mean result)/WEQAS SD at that level. SDI is a measure of total error and includes components of inaccuracy and imprecision. The four SDIs for the laboratory are used to calculate an overall analyte SDI, in this case 0.78. A value of less than 1 indicates that all estimates were within ±1 SD and is regarded as a good performance. A value greater than 2 would be indicative of an unacceptable performance.

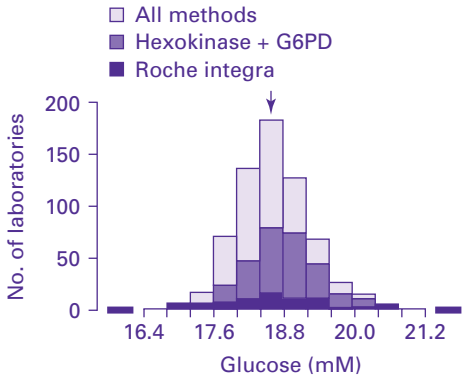

Fig. 1.7. Histogram of UK NEQAS quality assessment data for serum glucose based on data in Table 1.15a. The arrow indicates the location of the value submitted by the participating laboratory. (Reproduced by permission of UK NEQAS, Wolfson EQA Laboratory, Birmingham.)

clinical relevance of the tests performed, competence to carry out the tests as judged by the training and qualifications of the laboratory staff, health and safety, the quality of administrative and technical support systems and the quality of the laboratory management systems and document control. The successful outcome of an assessment is the national recognition that the laboratory is in compliance with the standards and hence provides quality healthcare. The accreditation normally lasts for 3 years.

1.8 SAFETY IN THE LABORATORY

Virtually all experiments conducted in a biochemistry laboratory present a potential risk to the wellbeing of the investigator. In planning any experiment it is essential that careful thought be given to all aspects of safety before the experimental design is finalised. Health hazards come from a variety of sources:

- *Chemical hazards:* All chemicals are, to varying extents, capable of causing damage to the body. First, they may be irritants and cause a short-term effect on exposure. Secondly, they may be corrosive and cause severe and often irreversible damage to the skin. Examples include strong acids and alkalis. Thirdly, they may be toxic once they have gained access to the body by ingestion, inhalation or absorption across the skin. Once in the body their effect may range from slight to the extremes of being a poison (e.g. cyanide), a carcinogen (e.g. benzene and vinyl chloride) or a teratogen (e.g. thalidomide). Finally there is the special case of the use of radioactive compounds that are discussed in detail in Chapter 14.
- *Biological hazards:* Examples include human body fluids that may carry infections such as the human immunodeficiency virus (HIV), laboratory animals that may cause allergic reactions or transmit certain diseases, pathogenic animal and cell tissue cultures, and all microorganisms, including genetically engineered forms. In the UK, animal experiments must be conducted in accordance with Home Office regulations and guidelines. All experiments with tissue and cell cultures should be conducted in microbiological cabinets that are provided with a sterile airflow away from the operator.
- *Electrical and mechanical hazards:* All electrical apparatus should be used and maintained in accordance with the manufacturers' instructions. Electrophoresis equipment presents a particular potential for safety problems. Centrifuges, especially high speed varieties, also need careful use especially in the correct use and balance of the rotors.
- *General laboratory hazards:* Common examples include syringe needles, broken glassware and liquid nitrogen flasks.

Routine precautions that should be taken to minimise personal exposure to these hazards include the wearing of laboratory coats, which should be of the high-necked buttoned variety for work with microorganisms, safety spectacles and lightweight disposable gloves. It is also good practice not to work alone in a

laboratory so that help is to hand if needed. In the UK, laboratory work is subject to legislation including the Health and Safety at Work Act, 1974, the *C*ontrol *of S*ubstances *H*azardous to *H*ealth (COSHH) Regulations 1994 and the Management of Health and Safety at Work Regulations 1999. This legislation requires a risk assessment to be carried out prior to laboratory work being undertaken. As the name implies, a risk assessment requires potential hazards to be identified and an assessment made of their potential severity and probability of occurrence. Action must be taken in cases where the potential severity and probability of an adverse event are medium to high. Such assessments require knowledge of the toxicity of all the chemicals used in the study. Toxicity data are widely available via computer packages and published handbooks and should be on reference in all laboratories. Once the toxicity data are known, consideration may be given to the use of alternative and less toxic compounds or, if it is decided to proceed with the use of toxic compounds, precautions taken to minimise their risk and plans laid for dealing with an accident should one occur. These include arranging access to first-aiders and other emergency services. It is normal for all laboratories to have a nominated Safety Officer whose responsibility it is to give advice on safety issues. To facilitate good practice, procedures for the disposal of organic solvents, radioactive residues, body fluids, tissue and cell cultures and microbiological cultures are posted in all laboratories. The outcome of the risk assessment is recorded on an approved form, signed by the investigator and countersigned by an approved senior person such as the Safety Officer. In all laboratory work it is essential to observe routine good practice including:

- always use an automatic pipette, such as the Gilson Pipetman^{R}, to pipette liquids: never be tempted to pipette by mouth, however, apparently innocuous the liquid;
- use a fume cupboard for potentially hazardous chemicals, ensuring that you conform to its correct operating procedure;
- use gas cylinders according to the suppliers specification;
- make sure you are aware of the location of safety equipment such as eyebaths and fire extinguishers;
- dispose of biological material, used glassware, pipette tips, broken glassware etc. according to laboratory procedures.

1.9 SUGGESTIONS FOR FURTHER READING

Analytical chemistry

BURNETT, D. (2002). *A Practical Guide to Accreditation in Laboratory Medicine*. ACB Ventures, London. (A comprehensive and highly user-friendly guide to this important aspect of quality assurance in clinical biochemistry.)

HARRIS, D. C. (2003). *Quantitative Chemical Analysis*, 6th edn. Freeman, New York. (An excellent undergraduate textbook covering all aspects of chemical analysis.)

KENKEL, J. (2000). *A Primer on Quality in the Analytical Laboratory*. Lewis, Boca Raton, FL. (A good introductory text on the general issues of quality control.)

SAUNDERS, G. C. and PARKES, H. C. (1999). *Analytical Molecular Biology*, LGC, Teddington, Middx. (Contains a very good chapter on quality in the molecular biology laboratory.)

Clinical biochemistry

BURTIS, C. A. and ASHWOOD, E. R. (2001). *Tietz Fundamentals of Clinical Chemistry*, 5th edn. Saunders, Philadelphia. (A comprehensive coverage of the principles and practice of clinical biochemistry.)

SMITH, A. F. and BECKETT, G. J. (1998). *Lecture Notes on Clinical Biochemistry*, 6th edn. Blackwell Science, Oxford. (An excellent reference text for all aspects of clinical biochemistry.)

Data analysis

JONES, R. and PAYNE, B. (1997). *Clinical Investigation and Statistics in Laboratory Medicine*. ACB Ventures, London. (Written specifically for analytical studies in clinical biochemistry.)

TOWNEND, J. (2002). *Practical Statistics for Environmental and Biological Scientists*. Wiley, Chichester. (A concise, user-friendly, non-technical introduction to statistics.)

Research methodology

BALNAVES, M. and CAPUTI, P. (2001). *Introduction to Quantitative Research Methods: An Investigative Approach*. Sage Publications, London. (A clear and accessible text with a practical emphasis based on case studies. Includes a CD-ROM for tutorial use.)

FESLING, M. F. W. (2003). Principles: The need for better experimental design. *Trends in Pharmacological Sciences*, **24**, 341–345. (Illustrates the importance of good experimental design by using a model experiment.)

Safety

Control of Substances Hazardous to Health Regulations 2002. Approved Code of Practice and Guidance. HSE Books, Kingston upon Thames. (A step-by-step approach to understanding the practical implications of COSHH.)

Chapter 2

Cell culture techniques

2.1 INTRODUCTION

Cell culture is a technique that involves the isolation and maintenance *in vitro* of cells isolated from tissues or whole organs derived from animals, microbes or plants. In general, animal cells have more complex nutritional requirements and usually need more stringent conditions for growth and maintenance. By comparison, microbes and plants require less rigorous conditions and grow effectively with the minimum of needs. Regardless of the source of material used, practical cell culture is governed by the same general principles, requiring a sterile pure culture of cells, the need to adopt appropriate aseptic techniques and the utilisation of suitable conditions for optimal viable growth of cells.

Once established, cells in culture can be exploited in many different ways. For instance, they are ideal for studying intracellular processes including protein synthesis, signal transduction mechanisms and drug metabolism. They have also been widely used to understand the mechanisms of drug actions, cell–cell interaction and genetics. Additionally, cell culture technology has been adopted in medicine, where genetic abnormalities can be determined by chromosomal analysis of cells derived, for example from expectant mothers. Similarly, viral infections can be assayed both qualitatively and quantitatively on isolated cells in culture. In industry, cultured cells are used routinely to test both the pharmacological and toxicological effects of pharmaceutical compounds. This technology thus provides a valuable tool to scientists, offering a user-friendly system that is relatively cheap to run and the exploitation of which avoids the legal, moral and ethical questions generally associated with animal experimentation.

In this chapter, fundamental information required for cell culture, together with a series of principles and outline protocols used routinely in growing animal, bacterial and plant cells are discussed. This should provide the basic knowledge for those new to the field of cell culture and act as a revision aid for those with limited experience in the field. Particular attention is paid to the importance of the work environment, outlining safety considerations together with adequate descriptions of the essential equipment required for tissue culture work.

2.2 THE CELL CULTURE LABORATORY AND EQUIPMENT

2.2.1 The cell culture laboratory

The design and maintenance of the cell culture laboratory is perhaps the most important aspect of cell culture, since a sterile surrounding is critical for handling of cells and culture media, which should be free from contaminating microorganisms. Such organisms, if left unchecked, would outgrow the cells being cultured, eventually resulting in culture-cell demise owing to the release of toxins and/or depletion of nutrient from the culture medium.

Where possible, a cell culture laboratory should be designed in such a way that it facilitates preparation of media and allows for the isolation, examination, evaluation and maintenance of cultures under controlled sterile conditions. In an ideal situation, there should be a room dedicated to each of the above tasks. However, many cell culture facilities, especially in academia, form part of an open plan laboratory and as such are limited in space. It is not unusual therefore to find an open plan area where places are designated for each of the above functions. This is not a serious problem as long as a few basic guidelines are adopted. For instance, good aseptic techniques (discussed below) should be used at all times. There should also be adequate facilities for media preparation, sterilisation and all cell culture materials should be maintained under sterile conditions until used. In addition, all surfaces within the culture area should be non-porous to prevent adsorption of media and other materials that may provide a good breeding ground for microorganisms, resulting in the infection of the cultures. Surfaces should also be easy to clean and all waste generated should be disposed of immediately. The disposal procedure may require prior autoclaving of the waste, which can be carried out using pressurized steam at 121 °C under 105 kPa for a defined period of time. These conditions are required to destroy microorganisms.

For smooth running of the facilities, daily checks should be made of the temperature in incubators, and of the gas supply to the incubators, by checking the CO_2 cylinder pressure. Water baths should be kept clean at all times and areas under the work surfaces of the flow cabinets cleaned of any spills.

2.2.2 Equipment for cell culture

Several pieces of equipment are essential. These include a tissue culture hood, incubator(s), autoclave and microscope. A brief description will be given of these and other essential equipments.

Cell culture hoods

The cell culture hood is the central piece of equipment where all the cell handling is carried out and is designed not only to protect the cultures from the operator but in some cases to protect the operator from the cultures. These hoods are generally referred to as laminar flow hoods as they generate a smooth uninterrupted streamlined flow (laminar flow) of sterile air which has been filtered through a high

efficiency particulate air (HEPA) filter. There are two types of laminar flow hood classified as either vertical or horizontal. The horizontal hoods allow air to flow directly at the operator and as a result are generally used for media preparation or when one is working with non-infectious materials including those derived from plants. The vertical hoods (also known as biology safety cabinets) are best for working with hazardous organisms, since air within the hood is filtered before it passes into the surrounding environment.

Currently, there are at least three different classes of hood used which all offer various levels of protection to the cultures, the operator or both and these are described below.

Class I hoods These hoods, as with the class II type, have a screen at the front that provides a barrier between the operator and the cells but yet allows access into the hood through an opening at the bottom of the screen (Fig. 2.1). This barrier prevents too much turbulence to air flow from the outside and, more importantly, provides good protection for the operator. Cultures are also protected but to a lesser extent when compared to the class II hoods as the air drawn in from the outside is sucked through the inner cabinet to the top of the hood. These hoods are suitable for use with low risk organisms and when operator protection only is required.

Class II hoods Class II hoods are the most common units found in tissue culture laboratories. These hoods offer good protection to both the operator and the cell culture. Unlike with class I hoods, air drawn from the outside is passed through the grill in the front of the work area and filtered through the HEPA filter at the top of the hood before streaming down over the tissue culture (Fig. 2.1). This mechanism protects the operator and ensures that the air over the cultures is largely sterile. These hoods are adequate for animal cell culture, which involves low to moderate toxic or infectious agents, but are not suitable for use with high-risk pathogens, which may require a higher level of containment.

Class III hoods Class III safety cabinets are required when the highest levels of operator and product protection are required. These hoods are completely sealed, providing two glove pockets through which the operator can work with material inside the cabinet (Fig. 2.1). Thus the operator is completely shielded, making class III hoods suitable for work with highly pathogenic organisms including tissue samples carrying known human pathogens.

Practical hints and safety aspects of using cell culture hoods All hoods must be maintained in a clutter free and clean state at all times as too much clutter may affect air flow and contamination will introduce infections. Thus, as a rule of thumb, put only items that are required inside the cabinet and clean all work surfaces before and after use with industrial methylated spirit (IMS). The latter is used at an effective concentration of 70% (prepared by adding 70% v/v IMS to 30% Milli-Q water),

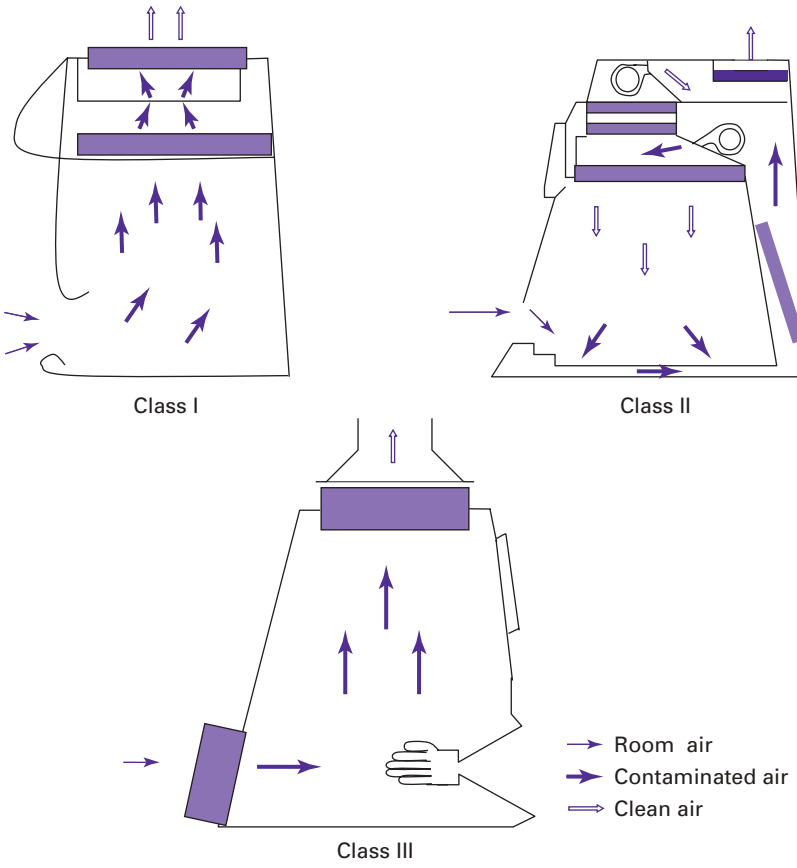

Fig. 2.1. Schematic representation of tissue culture cabinets.

which acts against bacteria and fungal spores by dehydrating and fixing cells, thus preventing contamination of cultures.

Some cabinets may be equipped with a short-wave ultraviolet light that can be used to irradiate the interior of the hood to kill microorganisms. When present, switch on the ultraviolet light for at least 15 min to sterilise the inside of the cabinet, including the work area. Note, however, that ultraviolet radiation can cause adverse damage to the skin and eyes and precaution should be taken at all times to ensure that the operator is not in direct contact with the ultraviolet light when using this option to sterilise the hood. Once finished, ensure that the front panel door (class I and II hoods) is replaced securely after use. In addition always turn the hood on for at least 10 min before starting work to allow the flow of air to stabilise. During this period, monitor the air flow and check all dials in the control panel at the front of the hood to ensure that they are within the safe margin.

CO_2 incubators

Water-jacketed incubators are required to facilitate optimal cell growth under strictly maintained and regulated conditions, normally requiring a constant temperature of 37 °C and an atmosphere of 5–10% CO_2 plus air. The purpose of the CO_2 is to ensure that the culture medium is maintained at the required physiological pH (usually pH 7.2–7.4). This is achieved by the supply of CO_2 from a gas cylinder into the incubator through a valve that is triggered to draw in CO_2 whenever the level fall below the set value of 5% or 10%. The CO_2 that enters the inner chamber of the incubator dissolves into the culture medium containing bicarbonate. The latter reacts with H^+ (generated from cellular metabolism), forming carbonic acid, which is in equilibrium with water and CO_2, thereby maintaining the pH in the medium at approximately pH 7.2.

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

These incubators are generally humidified by the inclusion of a tray of sterile water to the bottom deck. The evaporation of water creates a highly humidified atmosphere, which helps to prevent evaporation of medium from the cultures.

An alternative to humidified incubators is the dry non-gassed unit that is not humidified and relies on the use of alternative buffering systems such as 4(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) or morpholinopropane sulphonic acid (Mops) for maintaining a balanced pH within the culture medium. The advantage of this system is that it eliminates the risk from infections that can be posed by the tray of water in the humidified unit. The disadvantage, however, is that the culture medium will evaporate rapidly, thereby stressing the cells. One way round this problem is to place the cell culture plate in a sandwich box containing little pots of sterile water. With the sandwich box lid partially closed, evaporation of water from the pots will create a humidified atmosphere within the sandwich box, thus reducing the risk of evaporation of medium from the culture plate.

Practical hints and safety aspects of using cell culture incubators The incubator should be maintained at 37 °C and supplied with 5% CO_2 at all times. A constant temperature can be maintained by keeping a thermometer in the incubator, preferably on the inside of the inner glass door. This can then be checked on a regular basis and adjustments made as required. CO_2 levels inside the unit can be monitored and adjusted by using a gas analyser such as the Fryrite Reader. Regular checks should also be made on the levels of CO_2 in the gas cylinders that supply CO_2 to the incubators and when necessary replaced when levels are very low. Most incubators are designed with an in-built alarm that sounds when the CO_2 level inside the chamber drops. At this point the gas cylinder must be replaced immediately to avoid stressing or killing the cultures. It is now possible to connect two gas cylinders to a cylinder change-over unit that switches automatically to the second source of gas supply when the first is empty. It is advisable therefore to use this device where possible.

When one is using a humidified incubator, it is essential that the water tray is maintained and kept free from microorganisms. This can be achieved by adding various agents to the water such as the antimicrobial agent Roccal at a concentration of 1%(w/v). Other products such as Thimerosal or SigmaClean from Sigma-Aldrich can also be used. Proper care and maintenance of the incubator should, however, include regular cleaning of the interior of the unit using any of the above reagents then swabbed with 70% IMS. More recently, copper-coated incubators have been introduced which, due to the antimicrobial properties of copper, are reported to reduce microbial contamination.

Microscopes

Inverted phase contrast microscopes (see Chapter 4) are routinely used for visualising cells in culture. These are expensive but easy to operate, with a light source located above and the objective lenses below the stage on which the cells are placed. Visualisation of cells by microscopy can provide useful information about the morphology and state of the cells. Early signs of cells stress may be easily identified and appropriate action taken to prevent loss of cultures.

Other general equipment

Several other pieces of equipment are required in cell culture. These include a centrifuge to spin down cells, a water bath for thawing frozen samples of cells and warming media to 37 °C before use, and a fridge and freezer for storage of media and other materials required for cell culture. Some cells need to attach onto a surface in order to grow and are therefore referred to as adherent. These cells are cultured in non-toxic polystyrene plastics that contain a biologically inert surface on which the cells attach and grow. Various types of plastics are available for this purpose and include Petri dishes, multi-well plates (with either 96, 24, 12 or 6 wells per plate) and screwcap flasks classified according to their surface areas: T-25, T-75, T-225 (cm^2 of surface area). A selection of these plastics is shown in Fig. 2.2.

2.3 SAFETY CONSIDERATIONS IN CELL CULTURE

Because of the nature of the work, safety in the cell culture laboratory must be of a major concern to the operator. This is particularly the case when one is working with pathogenic microbes or with fresh primate or human tissues or cells that may contain agents that use humans as hosts. One very good example of this would be working with fresh human lymphocytes, which may contain infectious agents such as the human immunodeficiency virus (HIV) and/or hepatitis B virus. Thus, when one is working with fresh human tissue, it is essential that the infection status of the donor is determined in advance of use and all necessary precautions taken to eliminate or limit the risks to which the operator is exposed. A recirculation class II cabinet would be a minimum requirement for this type of cell culture work and the operator should be provided with protective

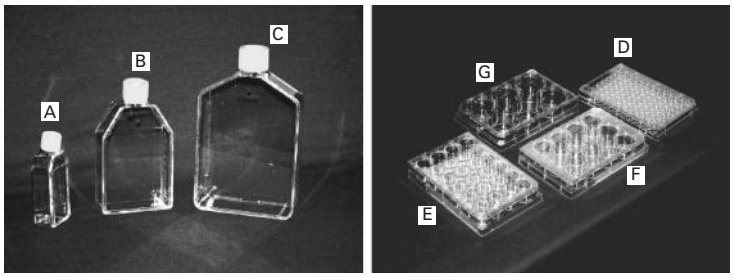

Fig. 2.2. Tissue culture plastics used generally for cell culture. (A–C) T-flasks; (D–G) representative of multi-well plates. (A) T–25 (25 cm^2), (B) T-75 (75 cm^2), (C) T–225 (225 cm^2), (D) 96-well plate, (E) 24-well plate, (F) 12-well plate, and (G) 6-well plate.

clothing including latex gloves and a face mask if required. Such work should also be carried out under the guidelines laid down by the UK Advisory Committee on Dangerous Pathogens (ACDP).

Apart from the risks posed by the biological material being used, the operator should also be aware of his or her work environment and be fairly conversant with the equipment being used, as these may also pose a serious hazard. The culture cabinet should be serviced routinely and checked (approximately every 6 months) to ensure its safety to the operator. Additionally the operator could ensure his or her own safety by adopting some common precautionary measures such as refraining from eating or drinking whilst working in the cabinet and using a pipette aid as opposed to mouth pipetting to prevent ingestion of unwanted substances. Gloves and adequate protective clothing such as a clean laboratory coat should be worn at all times and gloves must be discarded after handling of non-sterile or contaminated material.

2.4 ASEPTIC TECHNIQUES AND GOOD CELL CULTURE PRACTICE

2.4.1 Good practice

In order to maintain a clean and safe culture environment, adequate aseptic or sterile technique should be adopted at all times. This simply involves working under conditions that prevent contaminating microorganisms from the environment from entering the cultures. Part of the precaution taken involves washing hands with antiseptic soap and ensuring that all work surfaces are kept clean and sterile by swabbing with 70% IMS before starting work. Moreover, all procedures, including media preparation and cell handling, should be carried out in a cell culture cabinet that is maintained in a clean and sterile condition.

Other essential precautions should include avoiding talking, sneezing or coughing into the cabinet or over the cultures. A clean pipette should be used for each different procedure and under no circumstance should the same pipette be used between different bottles of media, as this will significantly increase the risk of cross-contamination. All spillages must be cleaned quickly to avoid

contamination from microorganisms that may be present in the air. Failing to do so may result in infections to the cultures, which may be reduced by using antibiotics. However, this is not always guaranteed and good aseptic techniques should eliminate the need for antibiotics. In the event of cultures becoming contaminated, these should be removed immediately from the laboratory, disinfected and autoclaved to prevent the contamination spreading. Under no circumstance can an infected culture be opened inside the cell culture cabinet or incubator. Moreover, all waste generated must be decontaminated and disposed of immediately after completing the work. This should be carried out in accordance with the national legislative requirements, which state that cell culture waste including media be inactivated using a disinfectant before disposal and that all contaminated materials and waste be autoclaved before being discarded or incinerated.

The risk from infections is the most common cause for concern in cell culture. Various factors can contribute to this, including poor work environment, poor aseptic techniques and indeed poor hygiene of the operator. The last of these is important, since most of the common sources of infections such as bacteria, yeast and fungus originate from the worker. Maintaining a clean environment and adopting good laboratory practice and aseptic techniques should, therefore, help to reduce the risks of infection. However, should infections occur, it is advisable to address this immediately and eradicate the problem. To do this, it helps to know the types of infection to expect and what to look for.

In animal cell cultures, bacterial and fungal infections are relatively easy to identify and isolate. The other most common contamination originates from mycoplasma. These are the smallest (approximately 0.3 μm in diameter) self-replicating prokaryotes in existence. They lack a rigid cell wall and generally infect the cytoplasm of mammalian cells. There are at least five species known to contaminate cells in culture: *Mycoplasma hyorhinis*, *Mycoplasma arginini*, *Mycoplasma orale*, *Mycoplasma fermentans* and *Acholeplasma laidlawii.* Infections caused by these organisms are more problematic and not easily identified or eliminated. Moreover, if left unchecked, mycoplasma contamination will cause subtle but adverse effects on cultures, including changes in metabolism, DNA, RNA and protein synthesis, morphology and growth. This can lead to non-reproducible, unreliable experimental results and unsafe biological products.

2.4.2 Identification and eradication of bacterial and fungal infections

Both bacterial and fungal contaminations are easily identified as the infective agents are readily visible to the naked eye even in the early stages. This is usually made noticeable by the increase in turbidity and the change in colour of the culture medium owing to the change in pH caused by the infection. In addition, bacteria can be easily identified under microscopic examination as motile round bodies. Fungi on the other hand are distinctive by their long hyphal growth and by the fuzzy colonies they form in the medium. In most cases the simplest solution to these infections is to remove and dispose of the contaminated cultures. In the

early stages of an infection, attempts can be made to eliminate the infecting microorganism using repeated washes and incubations with antibiotics or antifungal agents but this is not advisable as handling infected cultures in the sterile work environment increases the chances of the infection spreading.

As part of the good laboratory practice, sterile testing of cultures should be carried out regularly to ensure that cultures are free from microbial organisms. This is particularly important when preparing cell culture products or generating cells for storage. Generally, the presence of these organisms can be detected much earlier and necessary precautions taken to avoid a full-blown contamination crisis in the laboratory. The testing procedure usually involves culturing a suspension of cells or products in an appropriate medium such as tryptone soya broth (TSB) for bacterial or thioglycollate medium (TGM) for fungal detection. The mixture is incubated for up to 14 days but examined daily for turbidity, which is used as an indication of microbial growth. It is essential that both positive and negative controls are set up in parallel with the sample to be tested. For this purpose a suspension of bacteria such as *Bacillus subtilis* or fungus such as *Clostridium sporogenes* is used instead of the cells or product to be tested. Uninoculated flasks containing only the growth medium are used as negative controls. Any contamination in the cell cultures will result in the broth appearing turbid, as would the positive controls. The negative controls should remain clear. Infected cultures should be discarded, whilst clear cultures would be safe to use or keep.

2.4.3 Identification of mycoplasma infections

Mycoplasma contaminations are more prevalent in cell culture than many workers realise. The reason for this is that mycoplasma contaminations are not evident under light microscopy nor do they result in a turbid growth in culture. Instead the changes induced are more subtle and manifest themselves mainly as a slow-down in growth and in changes in cellular metabolism and functions. However, cells generally return to their native morphology and normal proliferation rates relatively rapidly after eradication of mycoplasma.

The presence of mycoplasma contamination in cultures has, until recently, been difficult to determine and samples had to be analysed by specialist laboratories. There are, however, improved techniques now available for detection of mycoplasma in cell culture laboratories. These involve either microbiological cultures of infected cells or an indirect DNA staining technique using the fluorochrome dye Hoechst 33258. With the former technique, cells in suspension are inoculated into liquid broth and then incubated under aerobic conditions at 37 °C for 14 days. A non-inoculated flask of broth is used as a negative control. Aliquots of broth are taken every 3 days and inoculated onto an agar plate, which is incubated anaerobically as above. All plates are then examined under an inverted microscope at a magnification of 300× after 14 days of incubation. Positive cultures will show the typical mycoplasma colony formation, which has an opaque granular central zone surrounded by a translucent border, giving a ‘fried egg’ appearance (Fig. 2.3). It may be necessary to set up positive controls in parallel,

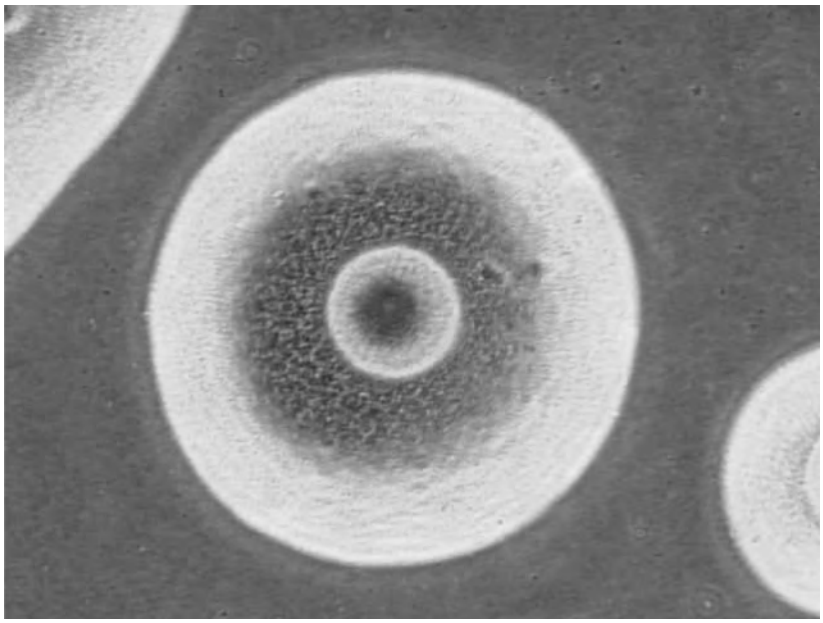

Fig. 2.3. Photograph of mycoplasma, showing the characteristic opaque granular central zone surrounded by a translucent border, giving a 'fried egg' appearance.

in which case plates and broth should be inoculated with a known strain of mycoplasma such as *Mycoplasma orale* or *Mycoplasma pneumoniae*.

The DNA binding method offers a rapid alternative for detecting mycoplasma and works on the principle that Hoechst 33258 fluoresces under ultraviolet light once bound to DNA. Thus, in contaminated cells, the fluorescence will be fairly dispersed in the cytoplasm of the cells owing to the presence of mycoplasma. In contrast, uncontaminated cells will show localised fluorescence in their nucleus only.

The Hoechst 33258 assay, although rapid, is relatively less sensitive when compared with the culture technique described above. For this assay, an aliquot of the culture to be tested is placed on a sterile coverslip in a 35 mm culture dish and incubated at 37 °C in a cell culture incubator to allow cells to adhere. The coverslip is then fixed by adding a fixative consisting of 1 part glacial acetic acid and 3 parts methanol, prepared fresh on the day. A freshly prepared solution of Hoechst 33258 stain is added to the fixed coverslip, incubated in the dark at room temperature to allow the dye to bind to the DNA and then viewed under ultraviolet fluorescence at 1000×. All positive cultures will show fluorescence of mycoplasma DNA, which will appear as small cocci or filaments in the cytoplasm of the contaminated cells (Fig. 2.4b, see colour section). Negative cultures will show only fluorescing nuclei of uncontaminated cells against a dark cytoplasmic background (Fig. 2.4a, see colour section). However, this technique is prone to errors, including false-negative results. To avoid the latter, cells should be cultured in antibiotic-free medium for two to three passages before being used. A positive control using a

strain of mycoplasma seeded onto a cover slip is essential. Such controls should be handled away from the cell culture laboratory to avoid contaminating clean cultures of cells. It is also important to ensure that the fluorescence detected is not due to the presence of bacterial contamination or debris embedded into the plastics during manufacture. The former normally appear larger than the fluorescing cocci or filaments of mycoplasma. Debris, on the other hand, would show a non-uniform fluorescence owing to the variation in size of the particles usually found in plastics.

2.4.4 Eradication of mycoplasma

Until recently, the most common approach for eradicating mycoplasma has been the use of antibiotics such as gentamycin. This approach is, however, not always effective, as not all strains of mycoplasma are susceptible to this antibiotic. Moreover antibiotic therapy does not always result in long-lasting successful elimination and most drugs can be cytotoxic to the cell culture. More recently, a new generation of bactericidal antibiotic preparation referred to as Plasmocin™ was introduced and has been shown to be effective against mycoplasma even at relatively low, non-cytotoxic concentrations. The antibiotics contained in this product are actively transported into cells, thus facilitating killing of intracellular mycoplasma but without any adverse effects on actual cellular metabolism.

Apart from antibiotics, various products have also been introduced into the cell culture market that the manufacturers claim eradicate mycoplasma efficiently and quickly without causing any adverse effects to the cells. One such product is Mynox®, a biological agent that integrates into the membrane of mycoplasma, compromising its integrity and eventually initiating its disintegration. This process apparently occurs within an hour of applying Mynox® and may have the added advantage that it is not an antibiotic and as a result will not lead to the development of resistant strains. It is safe to cultures and eliminated once the medium has been replaced. Moreover, this reagent is highly sensitive, detecting as little as 1–5 fg of mycoplasma DNA, which corresponds to two to five mycoplasma per sample and is effective against many of the common mycoplasma contaminations encountered in cell culture.

2.5 TYPES OF ANIMAL CELL AND THEIR CHARACTERISTICS IN CULTURE

The cell types used in cell culture fall into two categories generally referred to as either a primary culture or a cell line.

2.5.1 Primary cell cultures

Primary cultures are cells derived directly from tissues following enzymatic dissociation or from tissue fragments referred to as explants. These are usually the cells of preference, since it is argued that primary cultures retain their characteristics and reflect the true activity of the cell type *in vivo*. The disadvantage in using primary cultures, however, is that their isolation can be labour intensive and may

produce a heterogeneous population of cells. Moreover primary cultures have a relatively limited life span and can be used over only a limited period of time in culture.

Primary cultures can be obtained from many different tissues and the source of tissue used generally defines the cell type isolated. For instance, cells isolated from the endothelium of blood vessels are referred to as endothelial cells whilst those isolated from the medial layer of the blood vessels and other similar tissues are smooth muscle cells. Although both can be obtained from the same vessels, endothelial cells are different in morphology and function, generally growing as a single monolayer characterised by a cobble-stoned morphology. Smooth muscle cells on the other hand are elongated, with spindle-like projections at either end and grow in layers even when maintained in culture. In addition to these cell types there are several other widely used primary cultures derived from a diverse range of tissues, including fibroblasts from connective tissue, lymphocytes from blood, neurones from nervous tissues and hepatocytes from liver tissue.

2.5.2 Continuous cell lines

Cell lines consist of a single cell type that has gained the ability for infinite growth. This usually occurs after transformation of cells by one of several means that include treatment with carcinogens or exposure to viruses such as the monkey Simian virus 40 (SV40), Epstein–Barr virus (EBV) or Abelson murine leukaema virus (A-MuLV) amongst others. These treatments cause the cells to lose their ability to regulate growth. As a result, transformed cells grow continuously and, unlike primary culture, have an infinite life span (become 'immortalised'). The drawback to this is that transformed cells generally lose some of their original *in vivo* characteristics. For instance, certain established cell lines do not express particular tissue-specific genes. One good example of this is the inability of liver cell lines to produce clotting factors. Continuous cell lines, however, have several advantages over primary cultures, not least because they are immortalised. In addition, they require less serum for growth, have a shorter doubling time and can grow without necessarily needing to attach or adhere to the surface of the flask.

Many different cell lines are currently available from various cell banks, which makes it easier to obtain these cells without having to generate them. One of the largest organisations that supplies cell lines is the European Collection of Animal Cell Cultures (ECACC) based in Salisbury, UK. A selection of the different cell lines supplied by this organisation is listed in Table 2.1.

2.5.3 Cell culture media and growth requirements for animal cells

The cell culture medium used for animal cell growth is a complex mixture of nutrients (amino acids, a carbohydrate such as glucose, and vitamins), inorganic salts (e.g. containing magnesium, sodium, potassium, calcium, phosphate, chloride, sulphate, and bicarbonate ions) and broad-spectrum antibiotics. In

Table 2.1 Some commonly used cell lines supplied by cell banks

Cell line	Morphology	Species	Tissue origin
BAE-1	Endothelial	Bovine	Aorta
BHK-21	Fibroblast	Syrian hamster	Kidney
CHO	Fibroblast	Chinese hamster	Ovary
COS-1/7	Fibroblast	African green monkey	Kidney
HeLa	Epithelial	Human	Cervix
HEK-293	Epithelial	Human	Kidney
HT-29	Epithelial	Human	Colon
MRC-5	Fibroblast	Human	Lung
NCI-H660	Epithelial	Human	Lung
NIH/3T3	Fibroblast	Mouse	Embryo
THP-1	Monocytic	Human	Blood
V-79	Fibroblasts	Chinese hamster	Lung
HEP1	Hepatocytes	Human	Liver

certain situations it may be essential to include a fungicide such as amphotericin B, although this may not always be necessary. For convenience and ease of monitoring the status of the medium, the pH indicator phenol red may also be included. This will change from red at pH 7.2–7.4 to yellow or fuchsia as the pH becomes either acidic or alkaline, respectively.

The other key basic ingredient in the cell culture medium is serum, usually bovine or fetal calf. This is used to provide a buffer for the culture medium, but, more importantly, enhances cell attachment and provides additional nutrients and hormone-like growth factors that promote healthy growth of cells. An attempt to culture cells in the absence of serum does not usually result in successful or healthy cultures, even though cells can produce growth factors of their own. However, despite these benefits, the use of serum is increasingly being questioned not least because of many of the other unknowns that can be introduced, including infectious agents such as viruses and mycoplasma. The more recent resurgence of 'mad cow disease' (bovine spongioform encephalitis) has introduced an additional drawback, posing a particular risk for the cell culturist and has increased the need for alternative products. In this regard, several cell culture reagent manufacturers have now developed serum-free medium supplemented with various components including albumin, transferrin, insulin, growth factors and other essential elements required for optimal cell growth. This is proving very useful, particularly for the pharmaceutical and biotechnology companies involved in the manufacture of drugs or biological products for human and animal consumption.

2.5.4 Preparation of animal cell culture medium

Preparation of the culture medium is perhaps taken for granted as a simple straightforward procedure that is often not given due care and attention. As a

result, most infections in cell culture laboratories originate from infected media. Following the simple yet effective procedures outlined in Section 2.4.1 should prevent or minimise the risk of infecting the media when they are being prepared.

Preparation of the medium itself should also be carried out inside the culture cabinet and usually involves adding a required amount of serum together with antibiotics to a fixed volume of medium. The amount of serum used will depend on the cell type but usually varies between 10% and 20%. The most common antibiotics used are penicillin and streptomycin, which inhibit a wide spectrum of Gram-positive and Gram-negative bacteria. Penicillin acts by inhibiting the last step in bacterial cell wall synthesis whilst streptomycin blocks protein synthesis.

Once prepared, the mixture, which is referred to as complete growth medium, should be kept at 4 °C until used. To minimise wastage and risk of contamination it is advisable to make just the required volume of medium and use this within a short period of time. As an added precaution it is also advisable always to check the clarity of the medium before use. Any infected medium, which will appear cloudy or turbid, should be discarded immediately. In addition to checking the clarity, a close eye should also be kept on the colour of the medium, which should be red at physiological pH owing to the presence of phenol red. Media that looks acidic (yellow) or alkaline (fuchsia) should be discarded, as these extremes will affect the viability and thus growth of the cells.

2.5.5 Subculture of cells

Subculturing is the process by which cells are harvested, diluted in fresh growth medium and replaced in a new culture flask to promote further growth. This process, also known as passaging, is essential if the cells are to be maintained in a healthy and viable state, otherwise they may die after a certain period in continuous culture. The reason for this is that adherent cells grow in a continuous layer that eventually occupies the whole surface of the culture dish and at this point they are said to be confluent. Once confluent, the cells stop dividing and go into a resting state where they stop growing (senesce) and eventually die. Thus, to keep cells viable and facilitate efficient transformation, they must be subcultured before they reach full contact inhibition. Ideally, cells should be harvested just before they reach a confluent state.

Cells can be harvested and subcultured using one of several techniques. The precise method used is dependent to a large extent on whether the cells are adherent or in suspension.

Subculture of adherent cells Adherent cells can be harvested either mechanically, using a rubber spatula (also referred to as a rubber policeman) or enzymatically using proteolytic enzymes. Cells in suspension are simply diluted in fresh medium by taking a given volume of cell suspension and adding an equal volume of medium.

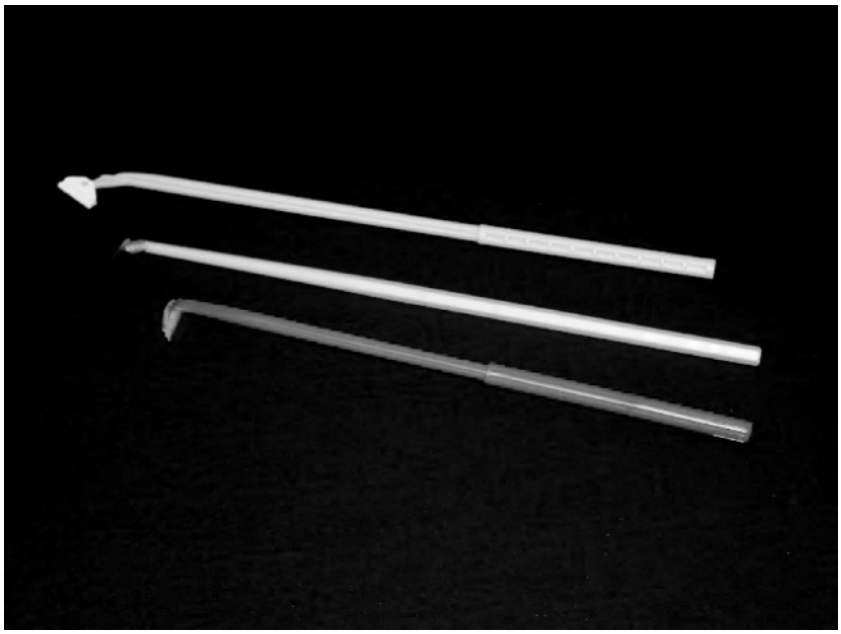

Fig. 2.5. Cell scrapers.

Harvesting of cells mechanically This method is simple and easy. It involves gently scraping cells from the growth surface into the culture medium using a rubber spatula that has a rigid polystyrene handle with a soft polyethylene scraping blade (Fig. 2.5). This method is not suitable for all cell types as the scraping may result in membrane damage and significant cell death. Before adopting this approach it is important to carry out some test runs where cell viability and growth are monitored in a small sample of cells following harvesting.

Harvesting of cells using proteolytic enzymes Several different proteolytic enzymes can be exploited including trypsin, a proteolytic enzyme that destroys proteinaceous connections between cells and between cells and the surface of the flask in which they grow. As a result, harvesting of cells using this enzyme results in the release of single cells, which is ideal for subculturing as each cell will then divide and grow, thus enhancing the propagation of the cultures.

Trypsin is commonly used in combination with EDTA, which enhances the action of the enzyme. EDTA alone can also be effective in detaching adherent cells as it chelates the Ca^{2+} required by some adhesion molecules that facilitate cell–cell or cell–matrix interactions. Although EDTA alone is much gentler on the cells than trypsin, some cell types may adhere strongly to the plastic, requiring trypsin to detach.

The standard procedure for detaching adherent cells using trypsin and EDTA involves making a working solution of 0.1% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline. The growth medium is aspirated from

confluent cultures and washed at least twice with a serum-free medium such as Ca^{2+} or Mg^{2+}-free PBS to remove traces of serum that may inactivate the trypsin. The trypsin-EDTA solution (approximately 1 cm^3 per 25 cm^2 of surface area) is then added to the cell monolayer and swirled around for a few seconds. Excess trypsin-EDTA is aspirated, leaving just enough to form a thin film over the monolayer. The flask is then incubated at 37 °C in a cell culture incubator for 2–5 min but monitored under an inverted light microscope at intervals to detect when the cells are beginning to round up and detach. This is to ensure that the cells are not overexposed to trypsin, as this may result in extensive damage to the cell surface, eventually resulting in cell death. It is important therefore that the proteolysis reaction is quickly terminated by the addition of complete medium containing serum that will inactivate the trypsin. The suspension of cells is collected into a sterile centrifuge tube and spun at 1000 r.p.m. for 10 min to pellet the cells, which are then resuspended in a known volume of fresh complete culture medium to give a required density of cells per cubic centimetre volume.

As with all tissue culture procedures, aseptic techniques should be adopted at all times. This means that all the above procedures should be carried out in a tissue culture cabinet under sterile conditions. Other precautions worth noting include the handling of the trypsin stock. This should be stored frozen at −20 °C and, when needed, placed in a waterbath just to the point where it thaws. Any additional time in the 37 °C waterbath will inactivate the enzymatic activity of the trypsin. The working solution should be kept at 4 °C once made and can be stored for up to 3 months.

Subculture of cells in suspension For cells in suspension it is important initially to examine an aliquot of cells under a microscope to establish whether cultures are growing as single cells or clumps. If cultures are growing as single cells, an aliquot is counted as described in Section 2.5.6 below and then reseeded at the desired seeding density in a new flask by simply diluting the cell suspension with fresh medium, provided the original medium in which the cells were growing is not spent. However, if the medium is spent and appears acidic, then the cells must be centrifuged at 1000 r.p.m. for 10 min, resuspended in fresh medium and transferred into a new flask. Cells that grow in clumps should first be centrifuged and resuspended in fresh medium as single cells using a glass Pasteur or fine-bore pipette.

2.5.6 Cell quantification

It is essential that when cells are subcultured they are seeded at the appropriate seeding density that will facilitate optimum growth. If cells are seeded at a lower seeding density they may take longer to reach confluency and some may expire before getting to this point. On the other hand, if seeded at a high density, cells will reach confluency too quickly, resulting in irreproducible experimental results. This is because trypsin can digest surface proteins, including receptors for drugs, and these will need time (sometimes several days) to renew. Failure to allow these

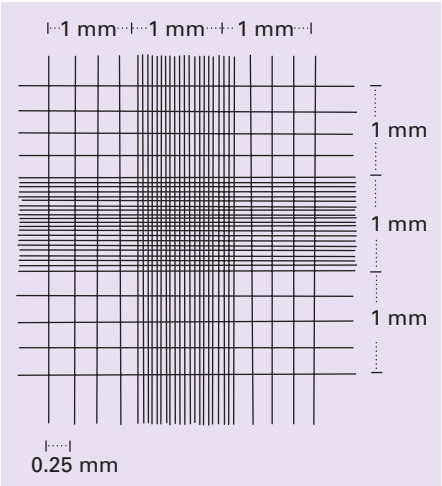

Fig. 2.6. Haemocytometer.

proteins to be regenerated on the cell surface may therefore result in variable responses to drugs specific for such receptors.

Several techniques are now available for quantification of cells and of these the most common method involves the use of a haemocytometer. This has the added advantage of being simple and cheap to use. The haemocytometer itself is a thickened glass slide that has a small chamber of grids cut into the glass. The chamber has a fixed volume and is etched into nine large squares, of which the large corner squares contain 16 small squares each; each large square measures 1 mm × 1 mm and is 0.1 mm deep (see Fig. 2.6).

Thus, with a coverslip in place, each square represents a volume of 0.1 mm^3 (1. 0 mm^2 area × 0. 1 mm depth) or 10^{-4} cm^3. Knowing this, the cell concentration (and the total number of cells) can therefore be determined and expressed per cubic centimetre. The general procedure involves loading approximately 10 μl of a cell suspension into a clean haemocytometer chamber and counting the cells within the four corner squares with the aid of a microscope set at 20× magnification. The count is mathematically converted to the number of cells/cm^3 of suspension.

To ensure accuracy, the coverslip must be firmly in place and this can be achieved by moistening a coverslip with exhaled breath and gently sliding it over the haemocytometer chamber, pressing firmly until Newton's refraction rings (usually rainbow-like) appear under the coverslip. The total number of cells in each of the four 1 mm^3 corner squares should be counted, with the proviso that only cells touching the top or left borders but not those touching the bottom and right borders are counted. Moreover, cells outside the large squares, even if they are within the field of view, should not be counted. When present, clumps should be counted as one cell. Ideally >100 cells should be counted to ensure a high degree of accuracy in counting. If the total cell count is less than 100 or if more

than 10% of the cells counted appear to be clustered, then the original cell suspension should be thoroughly mixed and the counting procedure repeated. Similarly, if the total cell count is greater than 400, the suspension should be diluted further to get counts of between 100 and 400 cells.

Since some cells may not survive the trypsinisation procedure it is usually advisable to add an equal volume of the dye trypan blue to a small aliquot of the cell suspension before counting. This dye is excluded by viable cells but taken up by dead cells. Thus, when viewed under the microscope, viable cells will appear as bright translucent structures while dead cells will stain blue (see Section 2.5.12). The number of dead cells can therefore be excluded from the total cell count, ensuring that the seeding density accurately reflects viable cells.

Calculating cell number

Cell number is usually expressed per cubic centimetre and is determined by multiplying the average of the number of cells counted by a conversion factor that is constant for each haemocytometer. The conversion factor is estimated at 1000, based on the fact that each large square counted represents a total volume of $10^{-4}\,cm^3$.
Thus:

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times \text{conversion factor}$$

If the cells were diluted before counting then the dilution factor should also be taken into account. Therefore:

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times \text{conversion factor} \times \text{dilution factor}$$

To find the total number of cells harvested, the number of cells determined per cubic centimetre should be multiplied by the original volume of fluid from which the cell sample was removed, i.e.:

$$\text{total cells} = \text{cells cm}^{-3} \times \text{total volume of cells}$$

Alternative methods for determination of cell number

Several other methods are available for quantifying cells in culture, including direct measurement using an electronic Coulter counter. This is an automated method of counting and measuring the size of microscopic particles. The instrument itself consists of a glass probe with an electrode that is connected to an oscilloscope (Fig. 2.7). The probe has a small aperture of fixed diameter near its bottom end. When immersed in a solution of cell suspension, cells are flushed through the aperture causing a brief increase in resistance owing to a partial interruption of current flow. This will result in spikes being recorded on the oscilloscope and each spike is counted as a cell. One disadvantage of this method, however, is that it does not distinguish between viable and dead cells.

Example 1 CALCULATION OF CELL NUMBER

Question

Calculate the total number of cells suspended in a final volume of 5 cm^3, taking into account that the cells were diluted 1:2 before counting and the number of cells counted with the haemocytometer was 400.

Answer

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{large squares counted}} \times \text{conversion factor}$$

$$= \frac{400}{4} \times 1000$$

$$= 100\,000 \text{ cells cm}^{-3}$$

Because there is a dilution factor of 2, the correct number of cells cm^{-3} is given as:

$100\,000 \times 2 = 200\,000$ cells cm^{-3}

Thus, in a final volume of 5 cm^3, the total number of cells present is:

$200\,000 \times 5 = 1\,000\,000$ cells

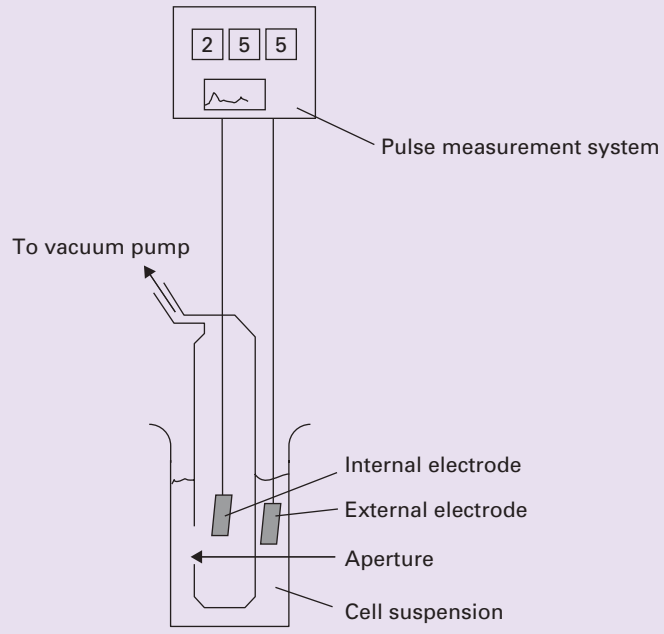

Fig. 2.7. Coulter counter. Cells entering the aperture create a pulse of resistance between the internal and external electrodes that is recorded on the oscilloscope.

Indirectly, cells can be counted by determining total cell protein and using a protein versus cell number standard curve to determine cell number in test samples. However, protein content per cell can vary during culture and may not give a true reflection of cell number. Alternatively, the DNA content of cells may be used as an indicator of cell number, since the DNA content of diploid cells is

usually constant. However, the DNA content of cells may change during the cell cycle and therefore not give an accurate estimate of cell number.

2.5.7 Seeding cells onto culture plates

Once counted, cells should then be seeded at a density that promotes optimal cell growth. It is essential therefore that when cells are subcultured they are seeded at the appropriate seeding density. If cells are seeded at a lower density they may take longer to reach confluency and some may die before getting to this point. On the other hand, if seeded at too high a density cells will reach confluency too quickly, resulting in irreproducible experimental results as already discussed above (see Section 2.5.6). The seeding density will vary depending on the cell type and on the surface area of the culture flask into which the cells will be placed. These factors should therefore be taken into account when deciding on the seeding density of any given cell type and the purpose of the experiments carried out.

2.5.8 Maintenance of cells in culture

It is important that after seeding, flasks are clearly labelled with the date, cell type and the number of times the cells have been subcultured or passaged. Moreover, a strict regime of feeding and subculturing should be established that permits cells to be fed at regular intervals without allowing the medium to be depleted of nutrients or the cells to overgrow or become super confluent. This can be achieved by following a standard but routine procedure for maintaining cells in a viable state under optimum growth conditions. In addition, cultures should be examined daily under a inverted microscope, looking particularly for changes in morphology and cell density. Cell shape can be an important guide when determining the status of growing cultures. Round or floating cells in subconfluent cultures are not usually a good sign and may indicate distressed or dying cells. The presence of abnormally large cells can also be useful in determining the well-being of the cells, since the number of such cells increases as a culture ages or becomes less viable. Extremes in pH should be avoided by regularly replacing spent medium with fresh medium. This may be carried out on alternate days until the cultures are approximately 90% confluent, at which point the cells are either used for experimentation or trypsinised and subcultured following the procedures outlined in Section 2.5.5.

The volume of medium added to the cultures will depend on the confluency of the cells and the surface area of the flasks in which the cells are grown. As a guide, cells which are under 25% confluent may be cultured in approximately 1 cm^3 of medium per 5 cm^2 and those between 25% and 40% or ≥45% confluency should be supplemented with 1. 5 cm^3 or 2 cm^3 culture medium per 5 cm^2, respectively. When changing the medium it is advisable to pipette the latter on either to the sides or the opposite surface of the flask from where the cells are attached. This is to avoid making direct contact with the monolayers as this will damage or dislodge the cells.

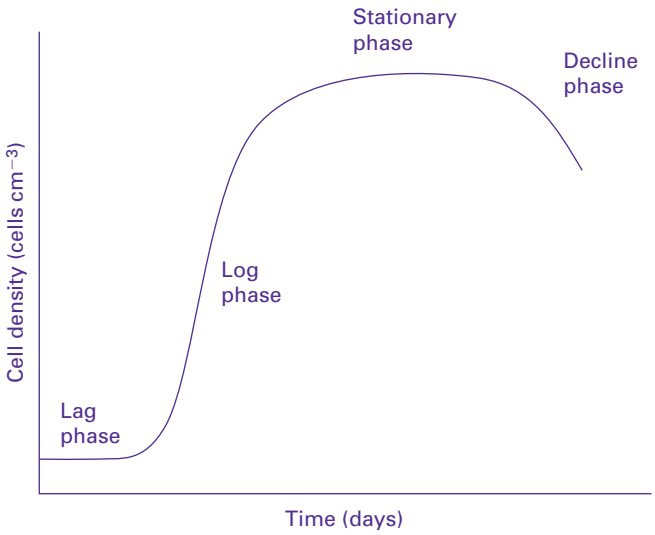

Fig. 2.8. Growth curve showing the phases of cell growth in culture.

2.5.9 Growth kinetics of animal cells in culture

When maintained under optimum culture conditions, cells follow a characteristic growth pattern (Fig. 2.8), exhibiting an initial lag phase in which there is enhanced cellular activity but no apparent increase in cell growth. The duration of this phase is dependent on several factors including the viability of the cells, the density at which the cells are plated and the media component.

The lag phase is followed by a log phase in which there is an exponential increase in cell number with high metabolic activity. These cells eventually reach a stationary phase where is there no further increase in growth due to depletion of nutrients in the medium, accumulation of toxic metabolic waste or a limitation in available growth space. If left unattended, cells in the stationary phase will eventually begin to die, resulting in the decline phase on the growth curve.

2.5.10 Cryopreservation of cells

Cells can be preserved for later use by freezing stocks in liquid nitrogen. This process is referred to as cryopreservation and is an efficient way of sustaining stocks. Indeed, it is advisable that, when good cultures are available, aliquots of cells should be stored in the frozen state. This provides a renewable source of cells that could be used in future without one necessarily having to culture new batches from tissues. Freezing can, however, result in several lethal changes within the cells, including formation of ice crystals and changes in the concentration of electrolytes and in pH. To minimise these risks a cryoprotective agent such as DMSO is usually added to the cells prior to freezing in order to lower the freezing point and prevent ice crystals from forming inside the cells. In addition, the

freezing process is carried out in stages, allowing the cells initially to cool down slowly from room temperature to −80 °C at a rate of 1–3 deg.C min^{-1}. This initial stage can be carried out using a freezing chamber or alternatively a cryo freezing container ('Mr Frosty') filled with isopropanol, which provides the critical, repeatable −1 deg.C min^{-1} cooling rate required for successful cell cryopreservation. When this process is complete, the cryogenic vials, which are polypropylene tubes that can withstand temperatures as low as −190 °C, are removed and immediately placed in a liquid nitrogen storage tank where they can remain for an indefinite period or until required.

The actual cryogenic procedure is itself relatively straightforward and involves harvesting cells as described in Section 2.5.5 and resuspending them in 1 cm^3 of freezing medium, which is basically culture medium containing 40% serum. The cell suspension is counted and appropriately diluted to give a final cell count of between 10^6 and 10^7 cells cm^{-3}. A 0.9 cm^3 aliquot is transferred into a cryogenic vial labelled with the cell type, passage number and date harvested. This is then made up to 1 cm^3 by adding 100 mm^3 of DMSO to give a final concentration of 10%. The cells should then be mixed gently by rotating or inverting the vial and placed in a 'Mr. Frosty' cryo freezing container. The container and cells should then be placed in a −80 °C freezer and allowed to freeze overnight. The frozen vials may then be transferred into a liquid nitrogen storage container. At this stage cells can be stored frozen until required for use.

All procedures should be carried out under sterile conditions to avoid contaminating cultures as this will appear once the frozen stocks are recultured. As an added precaution it is advisable to replace the growth medium in the 24 h period prior to harvesting cells for freezing. Moreover, cells used for freezing should be in the log phase of growth and not too confluent in case they may already be in growth arrest.

2.5.11 Resuscitation of frozen cells

When required, frozen stocks of cells may be revived by removing the cryogenic vial from storage in liquid nitrogen and placed in a waterbath at 37 °C for 1–2 min or until the ice crystals melt. It is important that the vials are not allowed to warm up to 37 °C as this may cause the cells to rapidly die. The thawed cell suspension may then be transferred into a centrifuge tube, to which fresh medium is added and centrifuged at 1000 r.p.m. for 10 min. The supernatant should be discarded to remove the DMSO used in the freezing process and the cell pellet resuspended in 1 cm^3 of fresh medium, ensuring that clumps are dispersed into single cells or much smaller clusters using a glass Pasteur pipette. The required amount of fresh pre-warmed growth medium is placed in a culture flask and the cells pipetted into the flask, which is then placed in a cell culture incubator and the cells allowed to adhere and grow.

2.5.12 Determination of cell viability

Determination of cell viability is extremely important, since the survival and growth of the cells may depend on the density at which they are seeded. The

degree of viability is most commonly determined by differentiating living from dead cells using the dye exclusion method. Basically, living cells exclude certain dyes that are readily taken up by dead cells. As a result, dead cells stain the colour of the dye used whilst living cells remain refractile owing to the inability of the dye to penetrate into the cytoplasm. One of the most commonly used dyes in such assays is trypan blue. This is incubated at a concentration of 0.4% with cells in suspension and applied to a haemocytometer. The haemocytometer is then viewed under an inverted microscope set at 100× magnification and the cells counted as described in Section 2.5.6, keeping separate counts for viable and non-viable cells.

The total number of cells is calculated using the following equation, as described previously:

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times \text{conversion factor} \times \text{dilution factor}$$

and the percentage of viable cells determined using the following formula:

$$\%\ \text{viability} = \frac{\text{number of unstained cells counted}}{\text{total number of cells counted}} \times 100$$

To avoid underestimating cell viability it is important that the cells are not exposed to the dye for more than 5 min before counting. This is because uptake of trypan blue is time sensitive and the dye may be taken up by viable cells during prolonged incubation periods. Additionally, trypan blue has a high affinity for serum proteins and as such may produce a high background staining. The cells should therefore be free from serum, which can be achieved by washing the cells with PBS before counting.

2.6 BACTERIAL CELL CULTURE

As with animal cells, pure bacterial cultures (cultures that contain only one species of organism) are cultivated routinely and maintained indefinitely using standard sterile techniques that are now well defined. However, since bacterial cells exhibit a much wider degree of diversity in terms of both their nutritional and environmental requirements, conditions for their cultivation are diverse and the precise requirements highly dependent on the species being cultivated. Outlined below are general procedures and precautions adopted in bacterial cell culture.

2.6.1 Safety considerations for bacterial cells culture

Culture of microbial cells, like that involving cells of animal origin, requires care and sterile techniques, not least of all to prevent accidental contamination of pure cultures with other organisms. More importantly, utmost care should be given

towards protecting the operator, especially from potentially harmful organisms. Aseptic techniques and safety conditions described for animal cell culture should be adopted at all times. Additionally, instruments used during the culturing procedures should be sterilised before and after use by heating in a Bunsen burner flame. Moreover, to avoid spread of bacteria, areas of work must be decontaminated after use using germicidal sprays and/or ultraviolet radiation. This is to prevent airborne bacteria from spreading rapidly. In line with these precautions, all materials used in microbial cell culture work must be disposed of appropriately; for instance, autoclaving of all plastics and tissue culture waste before disposal is usually essential.

2.6.2 Nutritional requirements of bacteria

The growth of bacteria requires much simpler conditions than those described for animal cells. However, due to their diversity, the composition of the medium used may be variable and largely determined by the nutritional classification of the organisms to be cultured. These generally fall into two main categories classified as either autotrophs (self-feeding organisms that synthesise food in the form of sugars using light energy from the sun) or heterotrophs (non-self-feeding organisms that derive chemical energy by breaking down organic molecules consumed). These in turn are subgrouped into chemo or photo autotrophs or heterotrophs. Both chemo- and photoautotrophs rely on carbon dioxide as a source of carbon but derive energy from completely different sources, with the chemoautotrophs utilising inorganic substances whilst the photoautotrophs use light. Chemoheterotrophs and photoheterotrophs both use organic compounds as the main source of carbon with the photoheterotrophs using light for energy and the chemo subgroup getting their energy from the metabolism of organic substances.

2.6.3 Culture media for bacterial cell culture

Several different types of medium are used to culture bacteria and these can be categorised as either complex or defined. The former usually consist of natural substances, including meat and yeast extract, and as a result are less well defined, since their precise composition is largely unknown. Such media are, however, rich in nutrients and therefore generally suitable for culturing fastidious organisms that require a mixture of nutrients for growth. Defined media, by contrast, are relatively simple. These are usually designed to the specific needs of the bacterial species to be cultivated and as a result are made up of known components put together in the required amounts. This flexibility is usually exploited to select or eliminate certain species by taking advantage of their distinguishing nutritional requirements. For instance, bile salts may be included in media when selective cultivation of enteric bacteria (rod-shaped Gram-negative bacteria such as *Salmonella* or *Shigella*) is required, since growth of most other Gram-positive and Gram-negative bacteria will be inhibited.

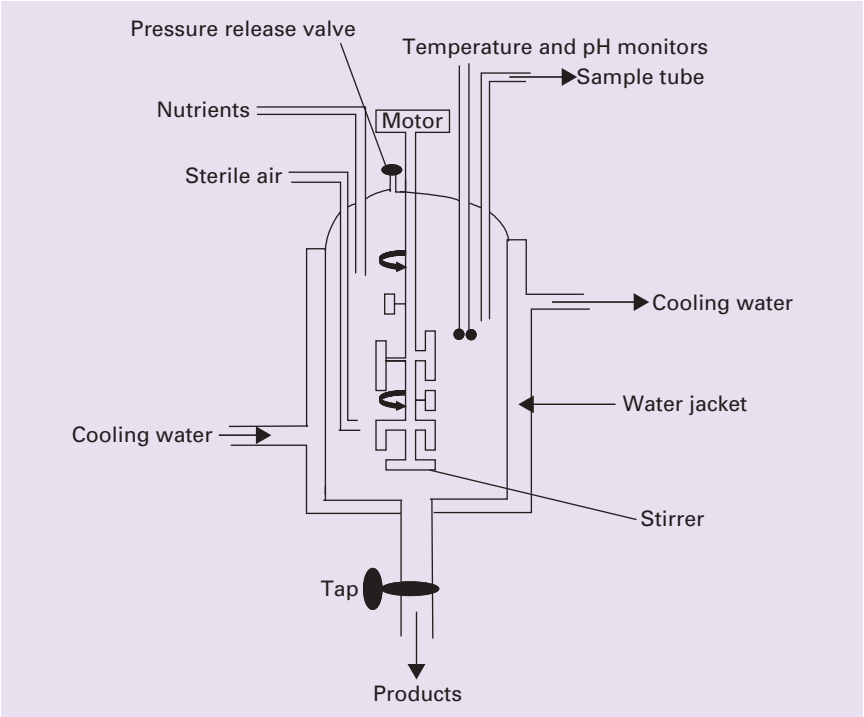

Fig. 2.9. Schematic representation of a fermenter.

2.6.4 Culture procedures for bacterial cells

Bacteria can be cultured in the laboratory using either liquid or solid media. Liquid media are normally dispensed into flasks and inoculated with an aliquot of the organism to be grown. This is then agitated continuously on a shaker that rotates in an orbital manner, mixing and ensuring that cultures are kept in suspension. For such cultures, sufficient space should be allowed above the medium to facilitate adequate diffusion of oxygen into the solution. Thus, as a rule of thumb, the volume of medium added to the flasks should not exceed more than 20% of the total volume of the flask. This is particularly important for aerobic bacteria and less so for anaerobic microorganisms.

In large-scale culture, fermenters or bioreactors equipped with stirring devices for improved mixing and gas exchange may be used. The device (Fig. 2.9) is usually fitted with probes that monitor changes in pH, oxygen concentration and temperature. In addition most systems are surrounded by a water jacket with fast flowing cold water to reduce the heat generated during fermentation. Outlets are also included to release CO_2 and other gases produced by cell metabolism.

When fermenters are used, precautions should be taken to reduce potential contamination with airborne microorganisms when air is bubbled through the cultures. Sterilisation of the air may therefore be necessary and can be achieved by

introducing a filter (pore size of approximately 0.2 μm) at the point of entry of the air flow into the chamber.

Solid medium is usually prepared by solidifying the selected medium with 1–2% of the seaweed extract agar, which, although organic, is not degraded by most microbes thereby providing an inert gelling medium on which bacteria can grow. Solid agar media are widely used to separate mixed cultures and form the basis for isolation of pure cultures of bacteria. This is achieved by streaking diluted cultures of bacteria onto the surface of an agar plate by using a sterile inoculating loop. Cells streaked across the plate will eventually grow into a colony, each colony being the product of a single cell and thus of a single species.

Once isolated, cells can be cultivated either in batch or continuous cultures. Of these, batch cultures are the most commonly used for routine liquid growth and entail inoculating an aliquot of cells into a sterile flask containing a finite amount of medium. Such systems are referred to as closed, since nutrient supply is limited to that provided at the start of culture. Under these conditions, growth will continue until the medium is depleted of nutrients or there is an excessive build-up of toxic waste products generate by the microbes. Thus, in this system, the cellular composition and physiological status of the cells will vary throughout the growth cycle.

In continuous cultures (also referred to as open systems) the medium is refreshed regularly to replace that spent by the cells. The objective of this system is to maintain the cells in the exponential growth phase by enabling nutrients, biomass and waste products to be controlled through varying the dilution rate of the cultures. Continuous cultures, although more complex to set up, offer certain advantages over batch cultures in that they facilitate growth under steady-state conditions in which there is tight coupling between cell division and biosynthesis. As a result, the physiological status of the cultures is more clearly defined, with very little variation in the cellular composition of the cells during the growth cycle. The main concern with the open system is the high risk of contamination associated with the dilution of the cultures. However, applying strict aseptic techniques during feeding or harvesting cells may help to reduce the risk of such contaminations. In addition, the whole system can be automated by connecting the culture vessels to their reservoirs through solenoid valves that can be triggered to open when required. This minimises direct contact with the operator or outside environment and thus reduces the risk of contamination.

2.6.5 Determination of growth of bacterial cultures

Several methods are available for determining the growth of bacterial cells in culture, including directly counting cells using a haemocytometer as described (Section 2.5.6). This is, however, suitable only for cells in suspension. When cells are grown on solid agar plates, colony counting can be used instead to estimate growth. This method assumes that each colony is derived from a single cell, which may not always be the case, since errors in dilution and/or streaking may result in clumps rather than single cells producing colonies. In addition, suboptimal culture

conditions may cause poor growth, thus leading to an underestimation of the true cell count. When cells are grown in suspension, changes in the turbidity of the growth medium could be determined using a spectrophotometer and the absorbance value converted to cell number using a standard curve of absorbance versus cell number. This should be constructed for each cell type by taking the readings of a series of known numbers of cells in suspension (see also Section 12.4.1).

2.7 PLANT CELL CULTURE

Plant cells, by being totipotent (i.e. capable of developing by regeneration into a whole new adult) are exploited in the laboratory for tissue culture purposes. Various techniques are used routinely to facilitate the regeneration of new plants from fully differentiated cells. This is achieved by inducing cells with various agents, including hormones (auxins, cytokinins), to undergo a new developmental programme and regenerate an entire new individual plant. As with all other tissue culture procedures, plant tissue culture requires a sterile environment, aseptic manipulation of specimens and defined growth conditions. The general laboratory requirements are similar to those outlined previously for animal cells but with certain modifications specific to plant tissue culture. For instance, a plant growth chamber or a controlled-environment room may be used instead of the standard CO_2 incubators required for animal cells. Other environmental conditions required for optimum growth will vary depending on the species but, in general, consideration should be given to the diurnal temperature variations, light quality and intensity and the relative length of light–dark cycles required by the plants.

2.7.1 Health and safety considerations for plant cell culture

The safety concerns in this case are relatively trivial when compared to the precautions that need to be taken when one is working with animal or bacterial cells. However, some plant tissues may pose a risk if contaminated with microorganisms and certain precautions should therefore be taken to prevent transfer or spread of the contaminant and, more importantly, protect the operator from inhaling spores. These should include surface sterilisation of the specimen using appropriate agents such as hypochlorite solution (1–10%, w/v). These agents pose an additional risk, as they can be harmful to the operator. Hypochlorite, for instance, can cause severe bronchial irritation if inhaled.

2.7.2 Plant culture media

Models for plant cell culture used *in vitro* involve incubating parts of a plant in a suitable medium under defined conditions. For this purpose, a wide range of different types of media that are chemically defined is now available from various commercial suppliers. Some of the most commonly used media include Murashige and Skoog, Gamborg B5, Nitsche, Shenck and Hildebrandt and McCown's Woody Plant Medium.

In addition to the balanced mixture, the medium may be supplemented with macro- (nitrogen, phosphorus, potassium, calcium, magnesium and sulphur) and micronutrients (iron, manganese, zinc, boron, copper, molybdenum and chlorine), vitamins (B vitamins) and a carbon source, which is usually sucrose or D-glucose. Growth regulators including cytokinins such as zeatin (naturally occurring) or its synthetic analogue 6-furfurylaminopurine (kinetin) may also be included to stimulate cell division. Some cultures may require auxin-like regulators that promote cell expansion and this may include the naturally occurring indol-3-yl acetic acid (IAA) or synthetic compounds such as 2,4-dichlorophenoxyacetic acid. Growth may be further enhanced by including oxygen-saturated perfluorochemicals (PFCs) such as perfluorodecalin (Flutec PP5), which improves oxygen supply to cells and thus stimulates growth. Non-ionic surfactants such as the polyoxyethylene–polypropylene copolymer Pluronic F68 are sometimes included in the media to increase plasma membrane permeability. Some media may contain activated charcoal (0–3%) or polyvinylpyrrolidone (PVP) and antioxidants to prevent browning due to phenol release, oxidation and polymerisation. Although antibiotics are not normally included, they may be employed when essential to clean up highly contaminated explants. Growth-retarding chemicals such as Paclobutrazol may also be added to the medium to ameliorate anatomical and physiological abnormalities associated with tissue incubation for long periods. Such verification is reduced when the medium is solidified with agar to approximately 1% (w/v) final concentration.

2.7.3 Plant cell culture systems

Setting up a plant cell or tissue culture begins with the excision and surface sterilisation of explants, which may be chosen from any part of the plant, depending on the objective of the study. Leaves are frequently preferred for protoplast isolation (cells without walls), anthers for production of haploids, shoot meristems for proliferating shoot cultures, and root tips for root cultures.

Explants

Explants may be excised either in the field or from glasshouse-grown plants or seedlings incubated under aseptic conditions. Surface sterilisation most frequently employs several minutes exposure to a 1–10% (w/v) sodium hypochlorite solution in which chlorine gas acts as a biocide. After the set time (of the order of 5 min) excess hypochlorite must be removed immediately by copious washing in sterile distilled water.

Explants are most likely to survive *in vitro* when the tissue chosen is physiologically active (i.e. not dormant). Many types of explant contain meristematic tissue capable of cell division. Undifferentiated callus is formed soon after excision and comprises cells with a small amount of cytoplasm but large vacuoles. Such tissue may develop localised growth centres called meristemoids from which caulogenesis (shoot indication), rhizogenesis (root initiation), or both may ensue. The ability of callus to undergo such organogenesis is genetically controlled but may be encouraged *in vitro* by manipulating the cytokinin to

auxin ratio in the medium. A high cytokinin to auxin ratio usually favours shoot proliferation, whereas a low cytokinin to auxin ratio usually promotes rooting. In practical terms, whole plantlets can be produced *in vitro* from organogenic callus by first encouraging shoot proliferation on a cytokinin-rich medium and then transferring leafy shoots to auxin-rich medium for root induction.

Roots may also be developed *in vivo*, provided plants are protected from desiccation. Organogenesis from callus is not generally recommended for plantlet multiplication *in vitro* (micropropagation) because there is considerable evidence that it results in genetically aberrant plants being recovered. A greater degree of chromosomal stability can be achieved by using shoot meristems as initial explants for reasons that are as yet ill defined. Meristem cultures are consequently a convenient starting material for micropropagation. Meristem culture can also be used sometimes to cure plants infected with viruses, following the use of high temperature treatment (thermotherapy) and/or chemicals (chemotherapy).

Calluses

Callus (an undifferentiated tissue formed at a wound in a plant) is often used as the starting material for suspension culture in which a mixture of both single cells and cell aggregates are incubated in a liquid medium that has to be artificially aerated to prevent the cells from becoming waterlogged. The ease of suspension formation is largely genetically controlled but friability can be improved empirically in certain cases by reducing calcium levels in the medium, altering the gas mix, changing the plant growth regulator regime or adding anti-oxidants or combinations of such variables.

Cell suspension

Cell suspension may grow as batch, fed-batch or continuous culture on a small, medium or large scale. For example, single individual cells may be grown in incubated microscope slide chambers where knowledge of the clonal origin of the plant is a prerequisite for the study. The wide-necked Erlenmeyer flask, shaken on a horizontal platform orbital shaker is a favourite container for small-scale batch culture. Fermenters with capacities of 1 to 50 000 dm^3 have been designed for large-scale cultures.

Cell suspensions provide excellent model systems for studies on cell division, cell expansion, cell differentiation and intermediary metabolism, because of the ease of adding test compounds and of harvesting cells and medium for analysis. The physiological properties of plant suspensions, however, render them more difficult to exploit than microbial cells. Moreover, the higher cost of plant culture medium, longer fermentation time (usually of the order of weeks), higher downstream processing costs and potential changes in the number of sets of chromosomes (i.e. ploidy instability) expressed in cells on prolonged subculture, significantly detracts from their usefulness for commercial exploitation (i.e. as important drugs, food additives, perfumes, biocides etc.). Despite these difficulties,

research is being conducted into using cell fermenters for plant secondary product synthesis because such compounds are either not possible or economically impractical to synthesise chemically. Since many desirable compounds are derived from plants found only in environmentally sensitive areas, there is added pressure on chemical manufacturers to turn to alternative sources of supply.

Plant cell culture suspensions are useful in experiments in which mutants are selected by growing colonies in the presence of the selecting agent. Ideally, suspensions comprising only single haploid cells should be used for mutant selection. Single-cell suspensions may be obtained by sequential filtration through a graded series of filters down to 50 μm pore size. Single-cell colonies selected for superior yields are usually transferred to a production medium, possibly involving mild stress, which suppresses cell division whilst promoting some degree of differentiation.

Protoplast

A protoplast is a spherical, osmotically sensitive cell with an intact cell membrane but lacking a cell wall. This can be prepared from whole cells following the removal of the cell wall either enzymatically using pectinase in conjunction with cellulases, or mechanically by dispersing plasmolysed tissues in which the protoplasts have shrunk away from the cell wall following incubations in a concentrated osmoticum such as mannitol (at approximately 500 mM). Of the two protocols, enzymatic isolations give much higher yields of uniform protoplast. However, incubation conditions must be carefully defined and monitored to avoid overexposure to the wall-degrading enzymes that may alter metabolism with deleterious consequences.

Protoplasts may be isolated from healthy leaves that have initially been sterilised in a solution of hypochlorite for approximately 10 min. The lower epidermis is peeled off and the leaf cut into small sections and incubated with the digesting enzyme mixture in the dark at 25 °C overnight. Protoplasts released into the suspension can then be separated from cell debris and purified by a combination of filtration using a fine nylon mesh, centrifugation at low speed and washing. The quality of the yield may be verified by checking their viability using dyes such as fluorescein diacetate or Evans Blue. The former is accumulated by viable protoplasts and subsequently converted by endogenous esterases to fluorescein, which can be visualised using ultraviolet microscopy. In contrast, Evans Blue is excluded by viable protoplasts and thus can be used to distinguish these from dead isolates.

Protoplasts will start to undergo division and form new calluses within 24 h of culture, following the regeneration of a new cell wall. To facilitate this, freshly isolated protoplasts should be counted to find an effective inoculum density for growth (10^4 cells cm^{-3}) prior to plating. As with previous counting procedures, this can be performed using a haemocytometer. For certain experimental purposes, protoplast fusion may be required. This may be particularly valuable in plant breeding experiments where sexual incompatibility between genera may be a limiting factor. Protoplast fusion can be induced by either chemical or physical means (electrofusion). Chemically, fusogens such as polyethyleneglycol (PEG,

approximately 30%) or a high pH calcium solution (1.1% (w/v) $CaCl_2.6H_2O$ in 10% (w/v) mannitol, pH 10.4) may be used to initiate this process, which can be achieved in less than 30 min.

Electrofusion is carried out in two steps. In the first, protoplasts are placed in a medium of low conductivity between two electrodes (platinum wires arranged in parallel on a microscope slide). A high frequency alternating field (0.5–1. 5 MHz) is applied between the electrodes, which causes the protoplasts to align in a process known as di-electrophoresis. In the second step, one or more short (10–200 μs) direct current pulses (of 1–3 $kV\,cm^{-1}$) are applied, which causes pores to form in the membranes of protoplasts and allows fusion to take place where there is close membrane contact. This technique allows a higher degree of control over the fusion process than do chemical methods.

2.8 POTENTIAL USE OF CELL CULTURES

Cell cultures of various sorts from animals, plants and microbes are becoming increasingly exploited not only by scientists for studying the activity of cells in isolation but also by various biotechnology and pharmaceutical companies for the production of valuable biological products including viral vaccines (e.g. polio vaccine), antibodies (e.g. OKT3 used in suppressing immunological organ rejection in transplant surgery) and various recombinant proteins. The application of recombinant DNA techniques has led to an ever-expanding list of improved products, from both mammalian and bacterial cells, for therapeutic use in humans. These products include the commercial production of factor VIII for haemophilia, insulin for diabetes, interferon-β and α for anticancer chemotherapy and erythropoietin for anaemia. Bacterial cultures have also been widely used for other industrial purposes, including the large-scale production of cell proteins, growth regulators, organic acids, alcohols, solvents, sterols, surfactants, vitamins, amino acids and many more products. In addition, degradation of waste products, particularly those from the agricultural and food industries, are another important industrial application of microbial cells. They are also exploited in the bioconversion of waste to useful end-products, and in toxicological studies where some of these organisms are rapidly replacing animals in preliminary toxicological testing of xenobiotics. More recently, both mammalian and bacterial cell systems are being developed to replace and supplement the use of animals in toxicological studies. Mammalian and bacterial cells are by no means the only systems being exploited. Plant cell cultures are used in genetic transformation and somatic hybridization, and protoplast fusion is proving especially valuable in plant breeding experiments, where it is used to overcome sexual incompatibility mechanisms between genera that prevent the formation of viable zygotes.

2.9 SUGGESTIONS FOR FURTHER READING

BALL, A. S. (1997). *Bacterial Cell Culture: Essential Data.* John Wiley & Sons, Inc., New York.
(Gives an adequate background into bacterial cell culture and techniques.)

DAVIS, J. M. (2002). *Basic Cell Culture: A Practical Approach*, 2nd edn. Oxford University Press, Oxford. (A comprehensive coverage of basic cell culture techniques.)

DIXON, R. A and GONZALES, R. A. (1994). *Plant Cell Culture: A Practical Approach*. Oxford University Press, Oxford. (Provides a good background on plant tissue culture techniques and application.)

FRESHNEY, R.I. (2000). *Culture of Animal Cells: A Manual of Basic Technique*, 4th edn. John Wiley & Sons, Inc., New York. (A comprehensive coverage of animal cell culture techniques and applications.)

FURR, A. K. (ed.) (1995). *CRC Handbook of Laboratory Safety*, 4th edn. CRC Press, Boca Raton, FL. (A complete guide to laboratory safety.)

HSC ADVISORY COMMITTEE ON DANGEROUS PATHOGENS (2001). *The Management Design and Operation of Microbiological Containment Laboratories*. HSE books, Sudbury. (Provides guidance, legal requirements and detailed technical information on the design, management and operation of containment laboratories.)

PAREKH, S. R. and VINCI, V. A. (2003). *Handbook of Industrial Cell Culture: Mammalian, Microbial, and Plant Cells*. Humana Press, Totowa, NJ. (Provides a good coverage of state-of-the-art techniques for industrial screening, cultivation and scale-up of mammalian, microbial, and plant cells.)