1 Basic principles

K. WILSON

1.1 BIOCHEMICAL AND MOLECULAR BIOLOGY STUDIES

1.1.1 Aims of laboratory investigations

Biochemistry involves the study of the chemical processes that occur in living organisms with the ultimate aim of understanding the nature of life in molecular terms. Biochemical studies rely on the availability of appropriate analytical techniques and on the application of these techniques to the advancement of knowledge of the nature of, and relationships between, biological molecules, especially proteins and nucleic acids, and cellular function. In recent years huge advances have been made in our understanding of gene structure and expression and in the application of techniques such as mass spectrometry to the study of protein structure and function. The Human Genome Project in particular has been the stimulus for major developments in our understanding of many human diseases especially cancer and for the identification of strategies that might be used to combat these diseases. The discipline of molecular biology overlaps with that of biochemistry and in many respects the aims of the two disciplines complement each other. Molecular biology is focussed on the molecular understanding of the processes of replication, transcription and translation of genetic material whereas biochemistry exploits the techniques and findings of molecular biology to advance our understanding of such cellular processes as cell signalling and apoptosis. The result is that the two disciplines now have the opportunity to address issues such as:

- the structure and function of the total protein component of the cell (*proteomics*) and of all the small molecules in the cell (*metabolomics*);
- the mechanisms involved in the control of gene expression;

- the identification of genes associated with a wide range of human diseases;
- the development of gene therapy strategies for the treatment of human diseases;
- the characterisation of the large number of 'orphan' receptors, whose physiological role and natural agonist are currently unknown, present in the human genome and their exploitation for the development of new therapeutic agents;
- the identification of novel disease-specific markers for the improvement of clinical diagnosis;
- the engineering of cells, especially stem cells, to treat human diseases;
- the understanding of the functioning of the immune system in order to develop strategies for the protection against invading pathogens;
- the development of our knowledge of the molecular biology of plants in order to engineer crop improvements, pathogen resistance and stress tolerance;
- the application of molecular biology techniques to the nature and treatment of bacterial, fungal and viral diseases.

The remaining chapters in this book address the major experimental strategies and analytical techniques that are routinely used to address issues such as these.

1.1.2 Experimental design

Advances in biochemistry and molecular biology, 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 which 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 evaluation of the outcomes including the application of appropriate statistical tests to quantitative data where applicable;
- 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.

The results of well-designed and analysed studies are finally published in the scientific literature after being subject to independent peer review, and one of the major challenges facing professional biochemists and molecular biologists is to keep abreast of current advances in the literature. Fortunately, the advent of the web has made access to the literature easier than it once was.

1.2 UNITS OF MEASUREMENT

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 Molarity – the expression of concentration

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 one cubic decimetre of solution. It is expressed by the symbol M.

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

Table 1.1 **SI units – basic and derived units**

Quantity	SI unit	Symbol (basic SI units)	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	$mol\,m^{-3}$		

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

Unit	Symbol	SI equivalent
Avogadro constant	L or N_A	$6.022 \times 10^{23}\ \text{mol}^{-1}$
Faraday constant	F	$9.648 \times 10^{4}\ \text{C mol}^{-1}$
Planck constant	h	$6.626 \times 10^{-34}\ \text{J s}$
Universal or molar gas constant	R	$8.314\ \text{J K}^{-1}\ \text{mol}^{-1}$
Molar volume of an ideal gas at s.t.p.		$22.41\ \text{dm}^3\ \text{mol}^{-1}$
Velocity of light in a vacuum	c	$2.997 \times 10^{8}\ \text{m s}^{-1}$
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	mm Hg	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

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

35.5 (Cl) i.e. 58.5, so that one mole is 58.5 grams. If this was dissolved in water and adjusted to a total volume of 1 dm^3 the solution would be one molar (1 M).

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 mM, μM and nM range rather than molar. Table 1.5 shows the interconversion of these units.

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

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 microlitre (μl)	10^{-3} ml	$= 1\ mm^{3}$	$= 10^{-9}\ m^{3}$
1 nanolitre (nl)	10^{-6} ml	$= 1\ nm^{3}$	$= 10^{-12}\ m^{3}$

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}

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. It also includes the reagents used in the preparation of buffers such as ethanoic (acetic) acid and phosphoric acid. 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, which 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

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 (K_{eq}) for the ionisation of water has a value of 1.8×10^{16} at 24 °C:

$$K_{eq} = \frac{[H^+][OH^-]}{[H_2O]} = 1.8 \times 10^{16} \quad (1.1)$$

The molarity of pure water is 55.6 M. This can be incorporated into a new constant, K_w:

$$1.8 \times 10^{-16} \times 55.6 = [H^+][HO^-] = 1.0 \times 10^{-14} = K_w \quad (1.2)$$

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 1.2 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 concentration, it follows that the pH of pure water is 7.0. This is the definition of neutrality.

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 which 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:

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

This reversible reaction can be represented by an equilibrium constant, K_a, known as the **acid dissociation constant** (equation 1.3). Numerically, it is very small.

$$K_a = \frac{[H^+][A^-]}{[HA]} \tag{1.3}$$

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 is represented by an equilibrium constant, K_b (equation 1.4):

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

$$K_b = \frac{[RNH_3^+][HO^-]}{[RNH_2][H_2O]} \tag{1.4}$$

In this case, the non-ionised form of the base abstracts a hydrogen ion from water to produce the conjugate acid that is ionised. If this equation is viewed from the reverse direction it is of a similar format to that of equation 1.3. Equally, equation 1.3 viewed in reverse is similar in format to equation 1.4.

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 in the following way:

$$K_a = \frac{[CH_3COO^-][H^+]}{[CH_3COOH]} = \frac{[\text{conjugate base}][H^+]}{[\text{weak acid}]} \tag{1.5a}$$

K_a has a value of 1.75×10^{-5} M. In practice it is far more common to express the K_a value in terms of its negative logarithm (i.e. $-\log K_a$) referred to as pK_a. Thus in this case pK_a is equal to 4.75. It can be seen from equation 1.3 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:

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

$$K_b = \frac{[CH_3COOH][HO^-]}{[CH_3COO^-]} = \frac{[\text{weak acid}][OH^-]}{[\text{conjugate base}]} \tag{1.5b}$$

K_b has a value of 1.77×10^{-10} M, hence its pK_b (i.e. $-\log K_b$) = 9.25.

Multiplying these two expressions together results in the important relationship:

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

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

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

hence

$$pK_a + pK_b = pK_w = 14 \tag{1.6}$$

This relationship holds for all acid–base pairs and enables one pK_a value to be calculated from knowledge of the other. Biologically important examples of conjugate acid–base pairs are lactic acid/lactate, pyruvic acid/pyruvate, carbonic acid/bicarbonate and ammonium/ammonia.

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* biochemical 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, as it is widely used as the basis of a buffer in the pH region of 6.70 (see below):

$$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^- \quad pK_a^1\ 1.96$$
$$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-} \quad pK_a^2\ 6.70$$
$$HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-} \quad pK_a^3\ 12.30$$

Example 1 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:

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

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

$$\text{pH} = 3.38$$

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:

$$\alpha = \frac{[\text{acetate}]}{[\text{acetate}] + [\text{acetic acid}]}$$
$$= \frac{4.18 \times 10^{-4}}{4.18 \times 10^{-4} + 0.01 - 4.18 \times 10^{-4}}$$
$$= \frac{4.18 \times 10^{-4}}{0.01}$$
$$= 4.18 \times 10^{-2} \text{ or } 4.18\%$$

Thus the majority of the acetic acid is present as the unionised form. If the pH is increased above 3.38 the proportion of acetate present will increase in accordance with the Henderson–Hasselbalch equation.

1.3.3 Buffer solutions

A buffer solution is one that resists a change in pH on the addition of either acid or base. They are of enormous importance in practical biochemical work as so many biochemical molecules are weak electrolytes so that their ionic status varies with pH so there is a need to stabilise this ionic status during the course of a practical experiment. In practice, a buffer solution consists of an aqueous mixture of a weak acid and its conjugate base. The conjugate base component would neutralise any hydrogen ions generated during an experiment whilst the unionised acid would neutralise any base generated. 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:

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\frac{[\text{conjugate base}]}{[\text{weak acid}]} \tag{1.7}$$

or

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\frac{[\text{ionised form}]}{[\text{unionised form}]}$$

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

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

or

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} + \log\frac{[\text{unionised form}]}{[\text{ionised form}]}$$

Table 1.6 lists some weak acids and bases commonly used in the preparation of buffer solutions. Phosphate, Hepes and Pipes are commonly used because of their optimum pH being close to 7.4. The buffer action and pH of blood is illustrated in Example 2 and the preparation of a phosphate buffer is given in Example 3.

Buffer capacity

It can be seen from the Henderson–Hasselbalch equations that when the concentration (or more strictly the activity) of the weak acid and base is equal, their ratio is one and their logarithm zero so that $\mathrm{pH} = \mathrm{p}K_\mathrm{a}$. The ability of 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.

$$\beta = \frac{\mathrm{d}b}{\mathrm{dpH}} = \frac{-\mathrm{d}a}{\mathrm{dpH}} \tag{1.9}$$

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 $\mathrm{p}K_\mathrm{a} \pm 1$.

Example 2 BUFFER ACTION AND pH OF BLOOD

The normal pH of blood is 7.4 and is maintained at this value by buffer action in particular by the action of HCO_3^- and CO_2 resulting from gaseous CO_2 dissolved in blood and the resulting ionisation of carbonic acid:

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

It is possible to calculate an overall equilibrium constant (K_{eq}) for these two consecutive reactions and to incorporate the concentration of water (55.6 M) into the value:

$$K_{eq} = \frac{[H^+][HCO_3^-]}{[CO_2]} = 7.95 \times 10^{-7} \quad \text{hence } pK_{eq} = 6.1$$

Rearranging:

$$pH = pK_{eq} + \log \frac{[HCO_3^-]}{[CO_2]}$$

When the pH of blood falls due to the metabolic production of H^+, these equilibria shift in favour of increased production of H_2CO_3 that in turn ionises to give increased CO_2 that is then expired. When the pH of blood rises, more HCO_3^- is produced and breathing is adjusted to retain more CO_2 in the blood thus maintaining blood pH. Some disease states may change this pH causing either acidosis or alkalosis and this may cause serious problems and in extreme cases, death. For example, obstructive lung disease may cause acidosis and hyperventilation alkalosis. Clinical biochemists routinely monitor patient's acid–base balance in blood, in particular the ratio of HCO_3^- and CO_2. Reference ranges for these at pH 7.4 are $[HCO_3^-] = 18.0 - 26.0$ mM and $pCO_2 = 4.6$–6.9 kPa, which gives $[CO_2]$ in the range of 1.20 mM.

Question A patient suffering from acidosis had a blood pH of 7.15 and $[CO_2]$ of 1.15 mM. What was the patient's $[HCO_3^-]$ and what are the implications of its value to the buffer capacity of the blood?

Answer Applying the above equation we get:

$$pH = pK_{eq} + \log \frac{[HCO_3^-]}{[CO_2]}$$
$$7.15 = 6.10 + \log \frac{[HCO_3^-]}{1.15}$$
$$1.05 = \log \frac{[HCO_3^-]}{1.15}$$

Taking the antilog of this equation we get $11.22 = [HCO_3^-]/1.15$

Therefore $[HCO_3^-] = 12.90$ mM indicating that the bicarbonate concentration in the patient's blood had decreased by 11.1 mM i.e. 47% thereby severely reducing the buffer capacity of the patient's blood so that any further significant production of acid would have serious implications for the patient.

Example 3 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:

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

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 1.7) gives:

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

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

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

Since the total concentration of the two species needs to be 0.1 M it follows that $[HPO_4^{2-}]$ 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 $0.067 \times 143 = 9.46$ g (Na_2HPO_4) and $0.033 \times 120 = 4.00$ g (NaH_2PO_4). These weights would be dissolved in approximately 800 cm^3 pure water, the pH measured and adjusted as necessary, and the volume finally made up to 1 dm^3.

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. Buffers are most commonly 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.

1.3.4 Measurement of pH – the pH electrode

The pH electrode is an example of an **ion-selective electrode** (ISE) that responds to one specific ion in solution, in this case the hydrogen ion. The 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 cations 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 determined by the hydrogen ion gradient across the membrane. Moreover, since the hydrogen ion concentration on the inside is constant (due to the use of 0.1 M hydrochloric acid) the observed potential is directly dependent upon the hydrogen ion concentration of the test solution. In practice a small **junction** or **asymmetry potential** (E^*) is also created in part as a result of linking the glass electrode to a 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 commonly using two standard buffers of known pH. For each 10-fold change in the hydrogen ion concentration 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 sensitivity of pH measurements is influenced by the prevailing absolute temperature.

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

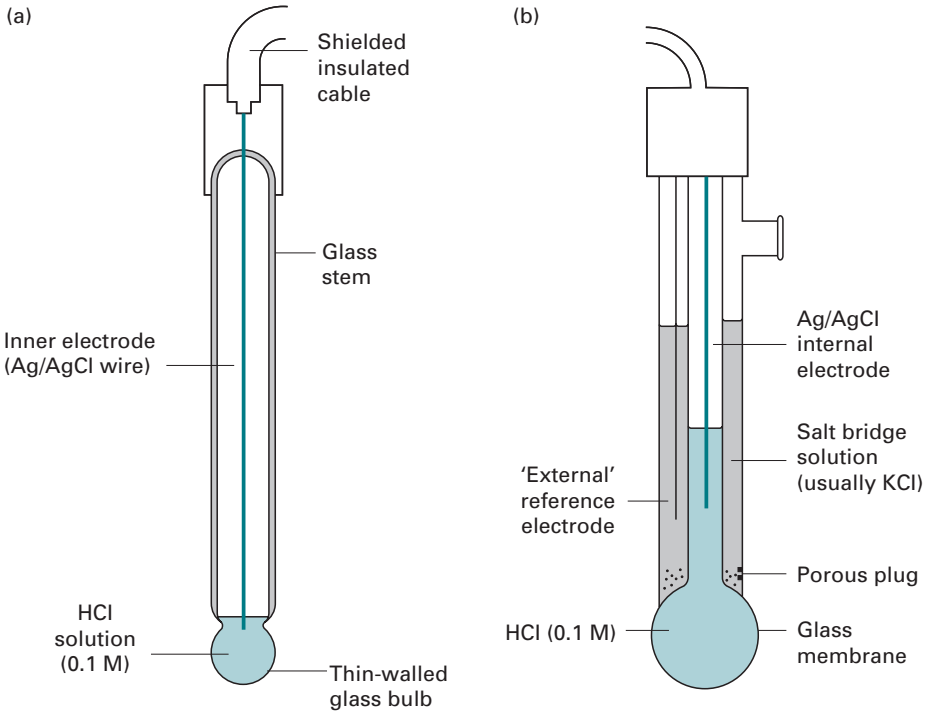

Fig. 1.1 Common pH electrodes: (a) glass electrode; (b) combination electrode.

1.3.5 Other electrodes

Electrodes exist for the measurement of many other ions such as Li^+, K^+, Na^+, Ca^{2+}, Cl^- and NO_3^- in addition to H^+. The principle of operation of these ion-selective electrodes (ISEs) is very similar to that of the pH electrode in that permeable membranes specific for the ion to be measured are used. They lack absolute specificity and their 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. Most ISEs have a good linear response to the desired ion and a fast response time. **Biosensors** are derived from ISEs by incorporating an immobilised enzyme onto the surface of the electrode. An important example is the glucose electrode that utilises glucose oxidase to oxidise glucose (Section 15.3.5) in the test sample to generate hydrogen peroxide that is reduced at the anode causing a current to flow that is then measured amperometrically. **Micro sensor** versions of these electrodes are of great importance in clinical biochemistry laboratories (Section 16.2.2). The **oxygen electrode** measures molecular oxygen in solution rather than an ion. It works by reducing the oxygen at the platinum cathode that is separated from the test solution by an oxygen-permeable membrane. The electrons consumed in the process are compensated by the generation of electrons at the silver anode hence the oxygen tension in the test sample is directly proportional to the current flow between the two electrodes. **Optical sensors** use the enzyme luciferase (Section 15.3.2) to measure ATP by generating light and detecting it with a photomultiplier.

1.4 QUANTITATIVE BIOCHEMICAL MEASUREMENTS

1.4.1 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, trans-membrane 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 them. This is particularly important in the field of clinical biochemistry in which quantitative measurements on a patient's blood and urine samples are used to aid a clinical diagnosis and monitor the patient's recovery from a particular disease. 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 required by the editors of 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.

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 HPLC and GC coupled to spectrophotometry or mass spectrometry, ion-selective electrodes and

immunological methods such as 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 re-assayed.

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 inbuilt 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 (see Section 16.2.3).

Random error (also called indeterminate error)

Random errors are caused by unpredictable and often uncontrollable inaccuracies in the various manipulations involved in the method. Such errors may be variably positive or negative and are caused by such factors 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 in 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 which must be followed.

1.4.2 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 *trueness*, *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 (see Section 16.1.2).
- *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 (see Section 1.4.6). 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 (see Section 16.1.2)

- *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 and 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.

1.4.3 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 alternative ways of expressing the scatter of the values within a data set about the **mean**, $\bar{x}$, 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 a measure of the variability of the population from which the data set was drawn. It is calculated by use of equation 1.10 or 1.11:

$$s = \sqrt{\frac{\Sigma(x_i - \overline{x})^2}{n-1}} \tag{1.10}$$

$$s = \sqrt{\frac{\Sigma x_i^2 - (\Sigma x_i)^2/n}{n-1}} \tag{1.11}$$

$(x_i - \bar{x})$ 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 out each other. 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 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 1.10 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 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 1.12.

$$CV = \frac{s100\%}{\bar{x}} \quad (1.12)$$

Since the mean and standard deviation have the same units, 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 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 (see Section 15.3.5) 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 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 shown the same precision for both samples. This illustrates the fact that 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 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%.

1.4.4 Assessment of accuracy

Population statistics

Whilst standard deviation and coefficient of variation give a measure of the variability 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 limit** 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 common 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 that the influence of random errors is minimised. In practice the population parameters are seldom measured for obvious practicality reasons and the sample parameters have

Example 4 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 (1.10) and (1.11) 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 1.10

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

Using equation 1.11

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

Coefficient of variation

Using equation 1.12

$$\text{CV} = \frac{0.165 \times 100\%}{2.42} = 6.82\%$$

Discussion In this case it is easier to appreciate the precision of the data set by considering the coefficient of variation. The value 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.

a larger uncertainty associated with them. The uncertainty of the sample mean deviating from the population mean decreases in the proportion of 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 two the number of experimental values would have to be increased four-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 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 limits 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 1.13):

$$\mu = \overline{x} \pm \frac{ts}{\sqrt{n}} \tag{1.13}$$

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 precision of the sample mean. Unlike standard deviation, standard error depends on the sample size and will fall as the sample size increases. The two measurements are sometimes confused, but in essence, standard deviation should be used if we want to know how widely scattered are the measurements and standard error should be used if we want to indicate the uncertainty around a mean measurement.

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 one in two 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 one in 20 or a one 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

Table 1.7 **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

(and hence with the varying degrees of freedom) at selected confidence levels are available in statistical tables. Some values are shown in Table 1.7. 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 some texts quote equation 1.13 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 are 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

Example 5 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 the previous worked example.

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' value lies.

As previously calculated, $\bar{x} = 2.42$ mM and $s = 0.16$ mM. Inspection of Table 1.8 reveals that for four degrees of freedom (the number of experimental values minus one) 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 to 2.47 mM, a 95% chance that the population mean lies within the range 2.22 to 2.62 mM and a 99% chance that it lies in the range 2.09 to 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 four-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 ± 0.07 mM 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.

Table 1.8 **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

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 1.8). 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 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 1.8 are based on a 95% confidence level.

The calculation of Q_{exp} is based upon equation 1.14 that 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:

$$Q_{exp} = \frac{x_n - x_{n-1}}{x_n - x_1} = \frac{\text{gap}}{\text{range}} \tag{1.14}$$

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

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

A *t*-test in general is used to address the question as to 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.

Example 6 IDENTIFICATION OF AN OUTLIER EXPERIMENTAL RESULT

Question If the data set in Example 5 contained an additional value of 3.0 mM, could this value be regarded as an outlier point at the 95% confidence level?

Answer From equation 1.15

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

Using Table 1.11 for six data points Q_{table} is equal to 0.62.

Since Q_{exp} is smaller than Q_{table} the point should not be rejected as there is more than a 95% 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.

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 measure 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 1.15. 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 1.7).

$$t_{calc} = \frac{(\text{known value} - \bar{x})\sqrt{n}}{s} \qquad (1.15)$$

These table values of t represent critical values that separate 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.

Example 7 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 (see Section 15.3.2 for details) 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 and 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 1.10 and 1.11 to the data set gives $\overline{x} = 5.15$ mM and $s = \pm 0.1$ mM

Now applying equation 1.16 to give t_{calc}:

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

Note that the negative difference between the two mean values in this calculation is ignored. From Table 1.10 at the 95% confidence level with four degrees of freedom, $t_{table} = 2.776$. t_{calc} is therefore less than t_{table} and the conclusion can be drawn that the measured mean value does agree with the known value. Using equation 1.13, the coefficient of variation for the measured values can be calculated to be 1.96%.

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, 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

Table 1.9 **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

or computer packages (Table 1.9). 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 1.16 and 1.17 are used to calculate t_{calc} for the two data sets. If not, equations 1.18 and 1.19 are used.

$$t_{calc} = \frac{\bar{x}_1 - \bar{x}_2}{s_{pooled}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \tag{1.16}$$

$$s_{pooled} = \sqrt{\frac{s_1^2(n_1 - 1) + s_2^2(n_2 - 1)}{n_1 + n_2 - 2}} \tag{1.17}$$

$$t_{calc} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(s_1^2/n_1 + s_2^2/n_2)}} \tag{1.18}$$

$$\text{degrees of freedom} = \left\{ \frac{(s_1^2/n_1 + s_2^2/n_2)^2}{(s_1^2/n_1)^2/(n_1 + 1) + (s_2^2/n_2)^2/(n_2 + 1)} \right\} - 2 \tag{1.19}$$

where $\bar{x}_1$ and $\bar{x}_2$ 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 ($\bar{x}$) and number of analytical measurements (n) and that the mathematical manipulation of the data is relatively easy.

Example 8 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 15.3.5). 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 we can calculate the mean, standard deviation and variance for each data set.

Glucose oxidase method

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

Hexokinase method

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

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

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

F_{table} for the two sets of data each with four 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 1.17 and 1.18 we can calculate that:

$$s_{\text{pooled}} = \sqrt{\frac{(0.16)^2(4) + (0.23)^2 4}{8}} = \sqrt{\frac{0.102 + 0.212}{8}} = \sqrt{0.039} = 0.198$$

$$t_{\text{calc}} = \frac{2.42 - 2.36}{0.198}\sqrt{\frac{(5)(5)}{10}} = (0.303)(1.58) = 0.48$$

Using Table 1.10 at the 95% confidence level and for eight 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.

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 1.20:

$$t_{calc} = \frac{\bar{d}}{s_d}\sqrt{n} \tag{1.20}$$

$$s_d = \sqrt{\frac{\Sigma(d_i - \bar{d})^2}{n-1}} \tag{1.21}$$

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.

1.4.6 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 ± one standard deviation 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, 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 $(\bar{x},\bar{y})$ where $\bar{x}$ and $\bar{y}$ 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

Example 9 COMPARISON OF TWO ANALYTICAL METHODS USING DIFFERENT TEST SAMPLES

Question Ten fasting serum samples were each analysed by the glucose oxidase and the 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		$\sum$ 0.3560

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

Answer Before addressing the main question, note that the ten 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 nine degrees of freedom in the study. To calculate t_{calc}, it is first necessary to calculate the value of s_d in equation 1.21. 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 1.20

$$t_{calc} = \frac{\bar{d}\sqrt{n}}{s_d}$$
$$= (0.12\sqrt{10})/0.199$$
$$= 1.907$$

Using Table 1.10, t_{table} at the 95% confidence level and for nine 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 seven of the ten samples analysed.

Example 9 (*cont.*)

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 one 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 to that of the hexokinase method.

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 one in which 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, e.g. 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.

1.4.7 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 the test analyte and which gives a similar response to the analytical method as 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 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 chromatographs are near to, but distinct from, the test analyte.

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 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.

1.5 SAFETY IN THE LABORATORY

Virtually all experiments conducted in a biochemistry laboratory present a potential risk to the well-being 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. They may be irritants and cause a short-term effect on exposure. Alternatively 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 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 (Section 2.2).
- *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 *Control of Substances Hazardous to Health (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 undertaking laboratory work. 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 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.

1.6 SUGGESTIONS FOR FURTHER READING

Analytical methodology and quality assurance

Burns, M. (2004). Current practice in the assessment and control of measurement uncertainty in bio-analytical chemistry. *Trends in Analytical Chemistry*, **23**, 393–397.

Carson, P. A. and Dent, N. (eds.) (2007). *Good Clinical, Laboratory and Manufacturing Practices: Techniques for the QA Professional*. London: RSC. (A comprehensive but easy-to-read book aimed at both newcomers and professionals involved in laboratory quality assurance issues.)

Fesling, M. F. W. (2003). Principles: the need for better experimental design. *Trends in Pharmacological Sciences*, **24**, 341–345.

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