$$\text{Rate} = k \times [A]^a \times [B]^b \qquad 4.5$$

where k is known as the rate constant.

The reaction is said to be of order a in A, of order b in B, and of overall order $a + b$. The order is an experimentally determined quantity and can have integral or non-integral values. For example, enzyme-catalysed reactions are of approximately first order with respect to substrate at low substrate concentrations, but the order decreases to near zero as the substrate concentration becomes saturating (see section 4.3.3).

The order of a reaction should not be confused with its molecularity, which is defined as the minimum number of species involved in the slowest step of the reaction, i.e. the minimum number of species involved in the transition state. The molecularity must therefore be a whole number.

The rate laws for reactions of different orders will be mentioned briefly and the method of analysis indicated.

Zeroth-order reactions are relatively rare. Examples in chemistry include some reactions of gases adsorbed on solid surfaces. In enzyme-catalysed reactions at very high substrate concentrations, the rate is close to zeroth order in substrate (see section 4.3.3).

Zeroth-order reactions: In this case, the rate does not depend on the concentration of the reactant A, i.e. rate of change of $[A] = -k$, where k is the rate constant. (The rate is negative because A is being consumed.) A plot of $[A]_t$ against time (Fig. 4.2) is a straight line of slope $-k$.

First-order reactions: In this case, the rate depends on $[A]$, i.e. rate of change of $[A] = k \times [A]$. Mathematical analysis shows that the concentration of A changes with time (t) as given by eqn. 4.6 (see Chapter 2, section 2.3.3):

$$[A]_t = [A]_0 \times e^{-kt} \qquad 4.6$$

By taking the natural logarithms of the terms on both sides of eqn. 4.6 and rearranging, we derive eqn. 4.7:

$$\ln [A]_t - \ln [A]_0 = -k \times t \qquad 4.7$$

where $[A]_0$ is the concentration of A at time $t = 0$, and k is the rate constant. A plot of $\ln [A]_t$ vs time (t) (Fig. 4.3) gives a straight line of slope $-k$.

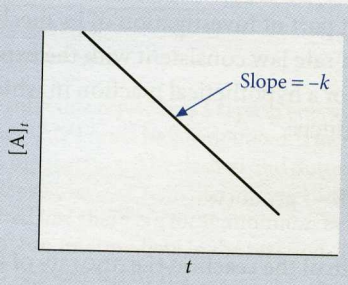


Fig. 4.2 Plot of $[A]_t$ vs. time for a zeroth-order reaction. The slope of the line is $-k$, where k is the rate constant.

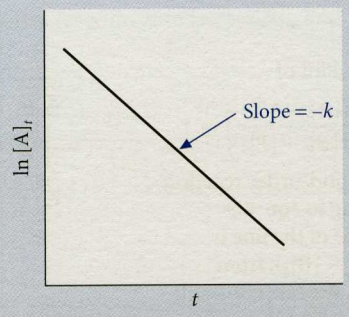


Fig. 4.3 Plot of ln $[A]_t$ against t for a first-order reaction according to eqn. 4.7. The slope of the line is $-k$, where k is the rate constant.

For a first-order reaction, the half-time $t_{1/2}$, i.e. the time taken for $[A]_t$ to decline to half its initial value, is found by setting $[A]_t = 0.5[A]_0$ in eqn. 4.6. This gives eqn. 4.8:

$$t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \qquad \textbf{4.8}$$

The constant values of successive half-times is a very convenient diagnostic test for a first-order reaction.

Note the half-time is independent of $[A]$, i.e. it takes as long for $[A]_t$ to fall from $[A]_0$ to $0.5[A]_0$ as it does for it to fall from $0.5[A]_0$ to $0.25[A]_0$.

Second-order reactions: If a reaction involves two reactants, A and B, whose initial concentrations are $[A]_0$ and $[B]_0$, respectively, then the concentration of product ($[P]$) formed after time t is given by eqn. 4.9:

$$\frac{1}{([A]_0 - [B]_0)} \times \ln\left(\frac{[B]_0([A]_0 - [P])}{[A]_0([B]_0 - [P])}\right) = k \times t \qquad \textbf{4.9}$$

where k is the rate constant.

A plot of $\ln\left(\dfrac{[B]_0([A]_0 - [P])}{[A]_0([B]_0 - [P])}\right)$ vs t (Fig. 4.4) will give a straight line of slope $k \times ([A]_0 - [B]_0)$, from which k can be determined.

The analysis of second-order reactions can be quite complex, but is simplified considerably under two conditions.

Condition 1 The first condition is where the concentrations of the two reactants are equal, i.e. $[A]_0 = [B]_0$. In this case, the concentration of product P after time t is given by eqn. 4.10:

$$\frac{[P]}{[A]_0([A]_0 - [P])} = k \times t \qquad \textbf{4.10}$$

so a plot of $\dfrac{[P]}{[A]_0([A]_0 - [P])}$ vs t (Fig. 4.5) will be a straight line of slope k.

Fig. 4.4 Plot of $\ln\left(\frac{[B]_0([A]_0-[P])}{[A]_0([B]_0-[P])}\right)$ vs. t for a second-order reaction according to eqn. 4.9. The slope of the line is $k\times([A]_0-[B]_0)$, from which k, the rate constant, can be derived.

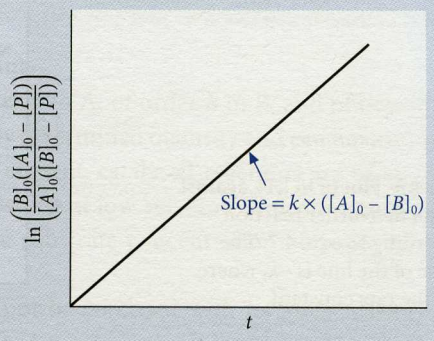
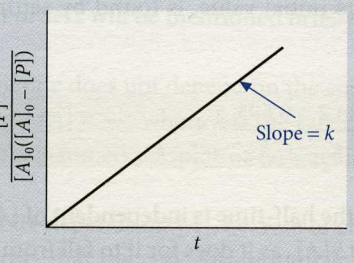


Fig. 4.5 Plot of $\frac{[P]}{[A]_0([A]_0-[P])}$ vs. t for a second-order reaction when $[A]_0=[B]_0$ according to eqn. 4.10. The slope of the line = k, where k is the rate constant.



The half-time of the reaction is derived from eqn. 4.10, by putting $[P]=0.5\,[A]_0$; this gives eqn. 4.11:

$$t_{1/2}=\frac{1}{k\times[A]_0} \qquad \textbf{4.11}$$

It is clear that each successive half-time becomes longer, i.e. it takes twice as long for $[A]$ to fall from $0.5\,[A]_0$ to $0.25\,[A]_0$ as it does from $[A]_0$ to $0.5\,[A]_0$.

WORKED EXAMPLE

Coenzyme A (denoted CoASH) which acts as a carrier of acyl groups in the metabolism of fatty acids and carbohydrates, contains a sulphydryl group. This group reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) with a 1:1 stoichiometry to give a yellow product (P), the thionitrophenolate ion which can be monitored by its absorbance at 412 nm. The data in Table 4.1 were obtained when equal concentrations (50 μM) of CoASH and Nbs_2 were reacted.

Table 4.1 Time course of the reaction between CoASH and Nbs_2

Time (min)	0	1	2	4	8	12	20	30
[P] (μM)	0	8.3	14.3	20.2	30.8	35.3	40.0	42.9

Show that the data conform to a second-order reaction with $[A]_0 = [B]_0$, and determine the second-order rate constant.

STRATEGY

This calculation involves evaluating the term $\frac{[P]}{[A]_0([A]_0 - [P])}$ at each time point to construct the appropriate plot. Use of the correct units will ensure that the slope is evaluated correctly.

SOLUTION

The plot of $\frac{[P]}{[A]_0([A]_0 - [P])}$ vs t (Fig. 4.6) is a straight line, showing that the reaction does obey second-order kinetics. The slope of the line corresponds to the second-order rate constant and is equal to 0.04 μM^{-1} min^{-1} = 4000 M^{-1} min^{-1} (note 1 $\mu M^{-1} = 10^6$ M^{-1}). This value can be seen to be reasonable by calculating the first-half life for the reaction using eqn. 4.11. Thus, $t_{1/2} = 1/k[A]_0 = 1/(4000 \times 50 \times 10^{-6})$ min = 5 min. Inspection of the data shows that $[P]$ has risen to half its final value (i.e. to 25 μM) between 4 and 8 min of reaction.

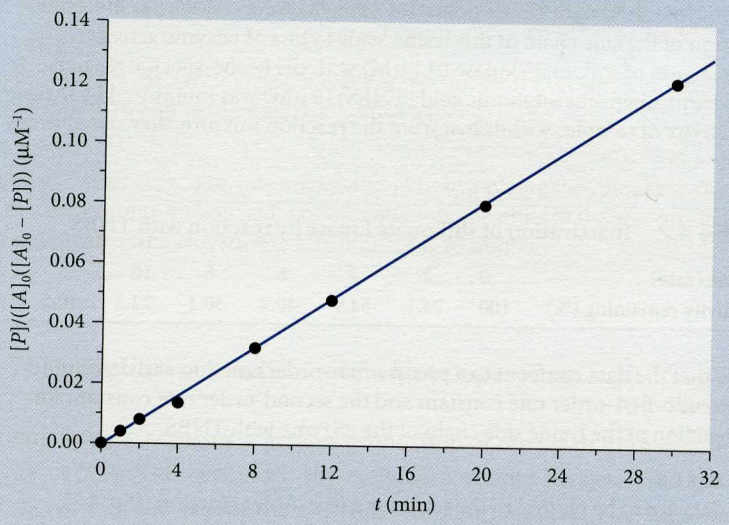


Fig. 4.6 Plot of $\frac{[P]}{[A]_0([A]_0 - [P])}$ vs. t for the reaction between CoA and Nbs_2 in the worked example according to eqn. 4.10. The slope of the line = k, where k is the rate constant.

Condition 2 The second condition under which the analysis of second-order reactions can be simplified is when the concentration of one of the reactants (say A) is much greater than that of the other (B). (In practice, this applies if the ratio $[A]_0/[B]_0$ is greater than about 20). If this applies, then the concentration of A remains essentially constant during the course of the reaction.

We can then write the rate of the reaction as eqn. 4.12:

$$\text{Rate} = k \times [A] \times [B] \qquad 4.12$$

but if $[A]$ remains effectively constant, this becomes eqn. 4.13:

$$\text{Rate} = k' \times [B] \qquad 4.13$$

where $k' = k \times [A]$.

Note it is important to express the pseudo-first-order rate constant and the second-order rate constant in the correct units (see section 4.2.2).

The reaction now behaves as a first-order reaction (it is termed a pseudo-first-order reaction) and can be analysed as such to give k', the pseudo-first-order rate constant. Division of k' by $[A]$, i.e. the concentration of the reactant in excess gives k, the second-order rate constant for the reaction.

WORKED EXAMPLE

The enzyme shikimate kinase possesses a single lysine residue per molecule. Reaction of the side chain of this lysine leads to loss of enzyme activity. The reaction of shikimate kinase (0.1 μM) with the lysine-specific reagent 2,4,6-trinitrobenzenesulphonic acid (TNBS) (5 μM) was monitored by measuring the activity of samples withdrawn from the reaction mixture after stated times (Table 4.2).

Table 4.2 Inactivation of shikimate kinase by reaction with TNBS

Time (min)	0	2	4	6	8	10	15	20
Activity remaining (%)	100	74.1	54.9	40.7	30.1	22.3	10.5	5.0

Show that the data conform to a pseudo-first-order reaction and determine the pseudo-first-order rate constant and the second-order rate constant for the reaction of the lysine side chain of the enzyme with TNBS.

STRATEGY

The data are to be plotted in the form for a first-order reaction (Fig. 4.3); if a straight line results, the slope can be used to calculate the rate constant.

SOLUTION

A plot of $\ln([A]_0/[A]_t)$ vs t is a straight line, showing that the loss in activity is occurring in a pseudo-first-order fashion. (Note the molar ratio of TNBS to enzyme is 50). The slope of the line $= -0.15\ \text{min}^{-1}$, so the pseudo-first-order rate constant (k') $= 0.15\ \text{min}^{-1}$. This is a reasonable value since it would correspond to a half-time for the reaction of $(\ln 2)/0.15\ \text{min} = 0.693/0.15\ \text{min} = 4.62\ \text{min}$. Inspection of the data shows that 50% of the original activity has been lost between 4 and 6 min of reaction. The second-order rate constant (k) is found by dividing k' by the concentration of TNBS (which is in excess). Hence $k = 0.15/(5 \times 10^{-6})\ \text{M}^{-1}\ \text{min}^{-1} = 30\,000\ \text{M}^{-1}\ \text{min}^{-1}$. It might be noted that this rate constant is much higher than for the reaction of the free amino acid lysine with TNBS and suggests that the side chain in the enzyme must be especially reactive.

4.2.2 The units of rates and rate constants

The rate of a reaction is measured in terms of the change in concentration of a reactant or product (depending on how the reaction is being monitored) with time. The units of rate will thus be concentration × (time)$^{-1}$, e.g. mM min^{-1}, μM s^{-1} etc.

The units of a rate constant will depend on the order of the reaction concerned, and can be deduced by taking into account the fact that units must balance on both sides of an equation (see Chapter 3, section 3.3).

Thus for a first-order reaction, rate = $k \times [A]$, so in terms of units we have:

left-hand side – the units are (concentration × (time)$^{-1}$)

right-hand side – the units are ((units of k) × concentration)

hence the units of k must be (time)$^{-1}$, e.g. s^{-1}, min^{-1}, etc.

WORKED EXAMPLE

What are the units of a second-order rate constant?

STRATEGY

From the rate law for a second-order reaction we can derive the units, noting that the units on the two sides of an equation must balance.

SOLUTION

For a second-order reaction, rate = $k \times [A] \times [B]$, so applying the rule about the need to balance units on both sides of the equation, the units of the second-order rate constant are (concentration)$^{-1}$ × (time)$^{-1}$, e.g. M min^{-1}.

4.2.3 The variation of reaction rate with temperature

As the temperature increases, a greater proportion of the reactant molecules have the energy necessary to surmount the activation energy barrier. This leads to a marked increase in the rate of the reaction with temperature. The equation proposed by Arrhenius (see Chapter 2, section 2.3.2) is:

$$k = A\, e^{-Ea/RT} \qquad 4.14$$

where k is the rate constant for the reaction, E_a is the activation energy, R is the gas constant (8.31 J K^{-1} mol^{-1}), and T is the temperature in degrees Kelvin. A is known as the pre-exponential factor and is related to the frequency of successful collisions between reacting molecules.

Taking natural logarithms of both sides of eqn. 4.14, we obtain eqn. 4.15:

$$\ln k = \ln A - \frac{E_a}{RT} \qquad 4.15$$

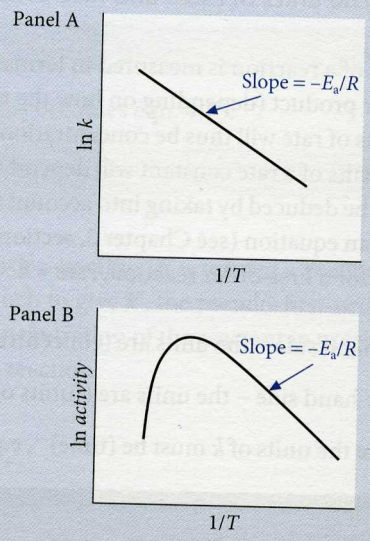


Fig. 4.7 Arrhenius plots to show the effect of temperature on reaction rate according to eqn. 4.15. (a) A typical plot for a chemical reaction. The slope of the line is $-E_a/R$, from which E_a can be derived. (b) A typical plot for an enzyme-catalysed reaction in which, above a certain temperature, there is a sharp loss of activity, reflecting the unfolding and inactivation of the enzyme.

Thus, an Arrhenius plot (ln k vs $1/T$) (Fig. 4.7) is a straight line of slope $-E_a/R$, from which E_a can be calculated.

In the case of enzyme-catalysed reactions, the rate will start to decline at higher temperatures because the three-dimensional structure of the enzyme which is required for the expression of catalytic activity will be disrupted by the loss of the weak interactions which stabilize it (see Chapter 1, section 1.7). The effect on the Arrhenius plot is shown in Fig. 4.7 (Panel B). The temperature at which the activity reaches a maximum value is often referred to as the 'optimum temperature', but this can be difficult to measure as it will depend on the length of time the enzyme is incubated at the temperature in question prior to assay. (For this reason, the term 'apparent optimum temperature' is often used.) In most cases the 'optimum temperature' of an enzyme will reflect the physiological needs of the organism in question. Some enzymes from hyperthermophilic bacteria, for example, seem to be stable for long periods at temperatures close to 100°C.

The availability of stable enzymes from thermophilic bacteria has been a key factor in their application in many processes. A good example is the use of the thermostable DNA polymerase from *Thermus aquaticus* in the polymerase chain reaction, which can amplify the amount of selected DNA by a very large factor (1 million-fold or greater).

WORKED EXAMPLE

The data in Table 4.3 were obtained for the activity of a sample of mammalian lactate dehydrogenase over a range of temperatures.

Table 4.3 The variation of lactate dehydrogenase activity with temperature

Temperature (°C)	5	15	25	35	45	55	65
Activity (μmol min^{-1} mg^{-1})	52.5	108	212	401	488	270	40.3

Explain the dependence of activity on temperature and determine the activation energy for the enzyme-catalysed reaction.

STRATEGY

The data can be plotted according to the Arrhenius equation to give a straight line (eqn. 4.15). Deviations from a straight line would indicate that additional factors have to be considered.

SOLUTION

The Arrhenius plot (ln (activity) vs $1/T$, where T is the temperature in degrees Kelvin = °C + 273, i.e. 25°C = 298 K) is linear over the range 5–35°C. The slope of the graph = −5810 K. The slope = $-E_a/R$, so the $E_a = 5810 \times 8.31$ J mol^{-1} = 48.3 kJ mol^{-1}. At 45°C, the rate is less than predicted on the basis of this straight line and at temperatures above 45°C, the activity declines sharply. This is due to progressive unfolding of the enzyme at the higher temperatures and the loss of catalytic activity. It should be noted that where a comparison can be made between an enzyme-catalysed reaction and the corresponding non-catalysed reaction, the activation energy of the latter is considerably greater.

SELF TEST ?

Check that you have mastered the key concepts at the start of the section by attempting the following questions.

ST 4.4 At low concentrations of substrate, the rate of an enzyme-catalysed reaction is given by: rate = $(k_{cat}/K_m) \times$ [Enzyme] × [Substrate]. At high concentrations of substrate, the rate is given by: rate = $k_{cat} \times$ [Enzyme]. Given that k_{cat} and K_m are constants, what is the order of the reaction under each condition?

ST 4.5 The dissociation of an antibody–antigen complex is a first-order reaction with a rate constant of 3×10^{-3} s^{-1}. What is the half-time for this reaction?

ST 4.6 The rate of a reaction catalysed by an enzyme from an intertidal species (winkle) increases by 1.2-fold as the temperature is raised from 5°C to 15°C. What is the activation energy for this reaction?

Answers

ST 4.4 The orders of the reaction are: at low [substrate], first order in enzyme and first order in substrate, second order overall; at high [substrate], first order in enzyme, zeroth order in substrate, first order overall.

ST 4.5 The half-time = 231 s, or 3 min 51 s.

ST 4.6 The activation energy = 12.1 kJ mol^{-1}.

Attempt Problems 4.5 and 4.6 at the end of the chapter.

In **ST 4.6**, the relatively small dependence of the rate with temperature is key to the survival of the organism, since the temperature will fluctuate considerably during the 24-h cycle of high and low tides.

4.3 Binding of ligands to macromolecules: saturation curves

KEY CONCEPTS

- Understanding the meaning of K_a and K_d in binding processes
- Understanding the relationship between K_d and ΔG^0
- Writing the equation for hyperbolic binding to single and multiple (independent) binding sites
- Writing the Hill equation to describe cooperative binding
- Understanding the meaning of V_{max} and K_m in enzyme-catalysed reactions
- Explaining the different types of inhibition of enzyme-catalysed reactions

Many biological processes depend on the interactions between molecules, for example regulation of gene expression (protein/DNA), catalysis (enzyme/substrate), cell signalling (hormone/receptor), etc. These interactions depend very largely on non-covalent forces (hydrogen bonds, van der Waals, electrostatic, hydrophobic interactions), which are individually weak but collectively can generate high affinities and specificities between the interacting molecules (for further details see Chapter 1, section 1.7). It is important to characterize binding processes to judge their importance under physiological conditions.

4.3.1 Single binding site on a macromolecule

For many of these interactions, simple saturation behaviour is observed:

$$P + L \rightleftharpoons PL$$

where P represents a protein and L a ligand, i.e. any molecule which binds to the protein; this could include a nucleic acid, a low molecular mass molecule or another protein, for example.

For this equilibrium, we can define two equilibrium constants, K_a and K_d, which are known as the association and dissociation constants, respectively:

$$K_a = \frac{[PL]}{[P] \times [L]} \qquad 4.16$$

$$K_d = \frac{[P] \times [L]}{[PL]} \qquad 4.17$$

where $[P]$ and $[L]$ represent the concentrations of free (i.e. unbound) protein and ligand, respectively, and $[PL]$ represents the concentration of the PL complex.

K_a represents a measure of the tendency for P and L to associate to form the PL complex. The larger this tendency, i.e. the tighter the binding, the greater the value of K_a.

K_d represents a measure of the tendency for the PL complex to dissociate into P and L. The smaller this tendency, i.e. the tighter the binding, the smaller the value of K_d.

For largely historical reasons, K_a is used predominantly by chemists and K_d by molecular biologists and biochemists. The two constants are reciprocals (see Chapter 2, section 2.4) of each other, i.e. $K_a = 1/K_d$ and $K_d = 1/K_a$. The units of K_a are (concentration)$^{-1}$, e.g. M^{-1}; those of K_d are concentration, e.g. M.

Strictly speaking, K_a and K_d are dimensionless since they refer to a standard state of a 1 M solution of P, L and PL (see section 4.1). It is convenient to keep the units of M^{-1} and M for K_a and K_d, respectively, to remember that for calculations, we must always express the concentrations in molar terms.

Since the equilibrium between P, L and PL is a dynamic one, at equilibrium the rate of the association reaction (P + L → PL), i.e. $k_a \times [P] \times [L]$, must equal the rate of the dissociation reaction (PL → P + L), i.e. $k_d \times [PL]$, where k_a and k_d are the association and dissociation rate constants, respectively. Thus, we have:

$$k_a \times [P] \times [L] = k_d \times [PL] \qquad 4.18$$

Hence, by combining eqn. 4.18 with eqns. 4.16 or 4.17, it follows that:

$$K_a = \frac{k_a}{k_d} \qquad 4.19$$

$$K_d = \frac{k_d}{k_a} \qquad 4.20$$

Thus, the equilibrium constants K_a and K_d can be expressed as the appropriate ratios of rate constants for the individual association and dissociation steps.

In order to develop molecular explanations for the interactions involving proteins, it is important to be able to link the value of K_a or K_d to the energy changes involved in binding or dissociation.

For the dissociation of the *PL* complex (PL ⇌ P + L), the relationship between the standard state free energy change (see section 4.1) and the value of K_d is given by eqn. 4.21 (which is analogous to eqn. 4.4).

$$-\Delta G^0 = RT \ln K_d \qquad 4.21$$

Thus, if $K_d = 1$ μM at 310 K (37°C), $\Delta G^0 = -8.31 \times 310 \times \ln (1 \times 10^{-6})$ J mol^{-1} = 35 600 J mol^{-1} = 35.6 kJ mol^{-1}. The energy change can be compared with typical values for the weak, non-covalent forces described in Chapter 1, section 1.7.

Note that K_d is expressed in terms of molar concentration, since this is referred to the standard state of the ligand, i.e. a 1 M solution.

In this example, the value of ΔG^0 is large and positive, since under standard state conditions (i.e. 1 M PL) there would be little tendency of the reaction to proceed from left to right, i.e. for PL to dissociate. Dissociation of the complex would be promoted by lowering the concentration of PL, which would be the case in living

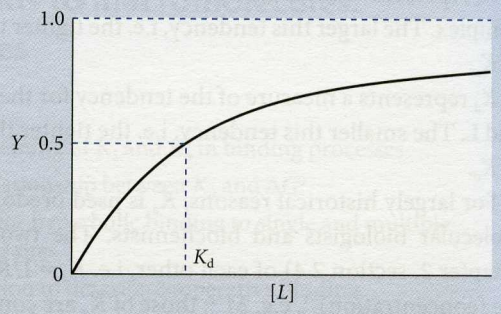


Fig. 4.8 A hyperbolic saturation curve for binding of a ligand (L) to a protein, according to eqn. 4.22. When the fractional saturation (Y) = 0.5, half the available sites are occupied, $[L] = K_d$.

systems, where concentrations of macromolecules are likely to be in the micromolar range, or even lower.

Remembering that at 310 K each factor of 10 in the value of K_d corresponds to approximately 6 kJ mol^{-1} (accurately 5.93 kJ mol^{-1}; see Chapter 2, section 2.3.6 and section 4.1 of this chapter) we can easily estimate the ΔG^0 involved. For example, a K_d of 10^{-9} M (1 nM) for a complex corresponds to a ΔG^0 of approximately 54 kJ mol^{-1} (accurately 53.4 kJ mol^{-1}).

It is a relatively simple task (see Appendix 4.1) to derive an expression for the fraction (Y) of the protein P which is saturated by ligand L (eqn. 4.22):

$$Y = \frac{[L]}{K_d + [L]} \qquad 4.22$$

The dependence of Y on $[L]$ according to eqn. 4.22 is described mathematically as a rectangular hyperbola (or simply a hyperbola). The binding is said to be hyperbolic in nature.

The value of Y can range from 0 to 1; a graph indicating how Y varies with $[L]$ is shown in Fig. 4.8.

Note that when half the total binding sites are occupied, i.e. $Y = 0.5$, then $K_d = [L]$. This gives a useful operational definition of K_d, namely that it corresponds to the concentration of (free) L at which half-saturation of the protein has occurred.

One of the problems about depicting the saturation curve as in Fig. 4.8 is that it is difficult to span a very wide range of ligand concentrations. For example, to go from 10% saturation to 90% saturation requires an 81-fold change in $[L]$ (from $K_d/9$ to $9 \times K_d$). The use of a logarithmic scale for $[L]$ can overcome this problem since it allows us to cover a very large numerical range of values (see Chapter 3, section 3.2). This type of plot is known as a formation plot and an example is shown in Fig. 4.9. In this figure the binding is hyperbolic (as in Fig. 4.8) but the logarithmic nature of the x-axis gives the curve a 'sigmoidal' (or elongated S-shape) appearance.

4.3.2 Multiple binding sites on a macromolecule

If instead of a single binding site on the protein, there are n sites for binding the ligand and these are assumed to be equivalent and independent of each other

$$P + nL \rightleftharpoons PL_n$$

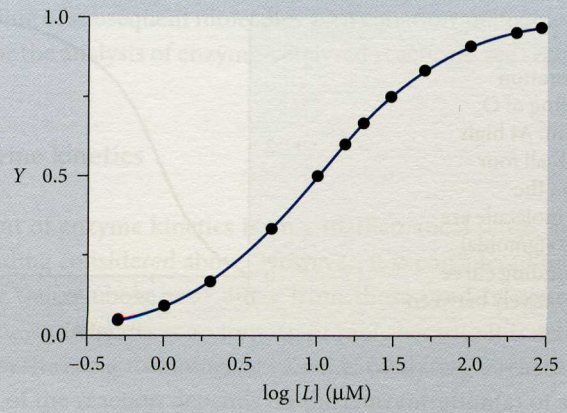


Fig. 4.9 Formation plot for binding of a ligand (L) to a protein, where [*L*] is plotted on a logarithmic scale. The data points correspond to a hyperbolic binding model (eqn. 4.22) with $K_d = 1$ μM. Note that in this example the use of the logarithmic scale allows data over a range of nearly 1000-fold values of [*L*] to be plotted.

then the saturation equation is analogous to eqn. 4.22, taking into account the number of binding sites; see eqn. 4.23:

$$r = \frac{n \times [L]}{K_d + [L]} \qquad 4.23$$

where r is the average number of molecules of ligand bound per molecule of protein, n is the number of binding sites, [*L*] is the concentration of free ligand, and K_d is the dissociation constant.

A plot of r against [*L*] is shown in Fig. 4.10. Note that r can take values ranging from 0 to n.

Substitution into eqn. 4.23 shows that when $r = 0.5n$ (i.e. when half the total sites are saturated), $[L] = K_d$. Thus, just as in the case of a single binding site, the value of

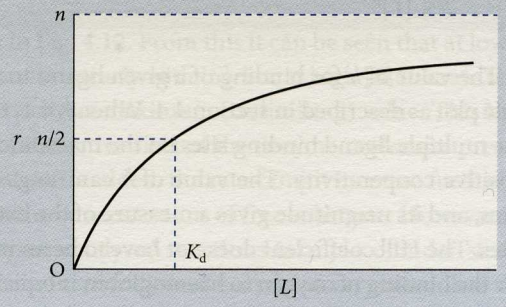


Fig. 4.10 Saturation curve for binding of a ligand (L) to n independent binding sites on a protein according to eqn. 4.23. When half the available sites are occupied (i.e. $r = n/2$), $[L] = K_d$.

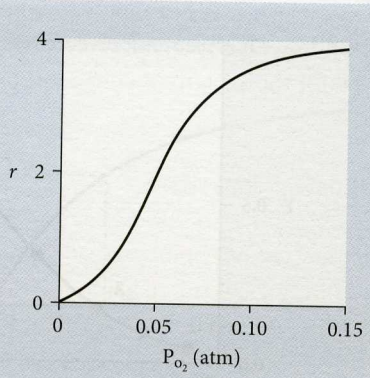


Fig. 4.11 Saturation curve for binding of O_2 to haemoglobin. At high pressures of O_2 all four binding sites in the haemoglobin molecule are occupied. The sigmoidal shape of the binding curve reflects cooperatively between the binding sites.

K_d is equal to the concentration of ligand required to bring about 50% saturation of the available sites.

Note in those cases where there are interactions between the binding sites, the shape of the saturation curve will differ. Thus, the binding of oxygen to haemoglobin (four binding sites) shows positive cooperativity, with the binding at the first site making it easier to bind to the subsequent sites. The saturation curve in this case is sigmoidal (see Fig. 4.11). This behaviour arises because of structural changes relayed between the subunits (polypeptide chains) of the protein.

Although a detailed mathematical analysis of cooperativity is beyond the scope of this book, mention should be made of the Hill equation (eqn. 4.24), which provides a relatively simple way of describing this type of behaviour. The equation is a modified form of eqn. 4.23 in which the term $[L]$ is raised to a power h, where h is known as the Hill coefficient.

Hill proposed this equation in 1910 to describe the binding of oxygen to haemoglobin.

$$r = \frac{n \times [L]^h}{K + [L]^h} \qquad 4.24$$

The Hill equation is usually expressed in terms of the fractional saturation (Y) of the binding sites by ligand, i.e. $Y = r/n$. Thus eqn. 4.24 becomes:

$$Y = \frac{[L]^h}{K + [L]^h} \qquad 4.25$$

The value of h for binding of a given ligand to a protein can be found by a suitable plot as described in section 4.4. When $h = 1$, there are no interactions between the multiple ligand binding sites on the macromolecule. A value of $h > 1$ indicates positive cooperativity. The value of h can range up to n, the number of binding sites, and its magnitude gives a measure of the extent of cooperativity between the sites. The Hill coefficient does not have to be an integer; for example the value of h for the binding of oxygen to haemoglobin is typically about 2.8. (A value of $h < 1$ is

said to indicate negative cooperativity, where the binding of the first ligand molecule hinders binding of subsequent molecules.) An equation analogous to eqn. 4.25 can be derived for the analysis of enzyme-catalysed reactions, see section 4.4.

4.3.3 Enzyme kinetics

The treatment of enzyme kinetics is, in a mathematical sense, analogous to that of ligand binding considered above. However, it is considered separately because a number of the symbols used differ from those used in considering binding equilibria. Enzyme kinetics is an important topic because it is actually measuring the enzyme performing its biological role (i.e. catalysing a reaction). By studying how the rate of the reaction depends on the concentration(s) of substrate(s), and the effect of inhibitor molecules (section 4.3.4) it is possible to draw conclusions not only about the mechanism of the reaction, but also about the possibilities of designing specific inhibitors which could act, for example as therapeutic drugs.

In the simplest case, where an enzyme (E) catalyses a reaction involving one substrate (S) and one product (P), the reaction is proposed to proceed via formation of an enzyme–substrate complex which breaks down to generate product and regenerate enzyme as follows:

$$E + S \rightleftharpoons ES \rightarrow E + P$$

By using either an equilibrium assumption (i.e. the breakdown of ES to give E + P is so slow as to not to perturb the $E + S \rightleftharpoons ES$ equilibrium significantly) or the steady-state assumption (i.e. the concentration of ES remains effectively constant once the steady-state condition has been achieved), we can derive an equation (4.26) to show how the rate (or velocity, v) varies with the concentration of substrate ($[S]$). This is known as the Michaelis–Menten equation.

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad 4.26$$

where V_{max} is the maximum rate (more correctly, the limiting rate) and K_m is known as the Michaelis constant. From eqn. 4.26 it can be seen that if v is set equal to 0.5 V_{max}, $[S] = K_m$.

The plot of v vs. $[S]$ is shown in Fig. 4.12. From this it can be seen that at low $[S]$ the rate of reaction increases almost linearly with $[S]$, but at high $[S]$ the rate tends to level off somewhat and approaches a maximum (or limiting) value. This levelling off reflects the saturation of the active sites of the enzyme molecules present; no matter how much more substrate is added, the rate cannot increase further.

Strictly speaking $[S]$ refers to the concentration of free substrate. However, the vast majority of studies of enzyme-catalysed reactions take place under steady-state conditions where the molar concentration of enzyme is very much less than

The derivation of the Michaelis–Menten equation is discussed in more detail in Appendix 4.1.

The Michaelis–Menten equation was derived in 1913 by Leonor Michaelis (Belgian) and Maud Menten (Canadian) using the equilibrium assumption. In 1925, Briggs and Haldane (both British) applied the steady-state approximation to derive an equation of similar form.

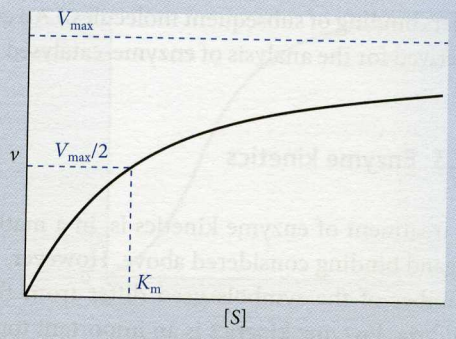


Fig. 4.12 Michaelis–Menten plot showing the variation of the velocity (rate) of an enzyme-catalysed reaction with the concentration of substrate according to eqn. 4.26. When $v = V_{max}/2$, $[S]$ = the Michaelis constant (K_m).

the molar concentration of substrate. In these circumstances, the concentration of free substrate is effectively equal to that of the total added substrate.

It should be noted that when E and S are mixed the system takes a short time to reach the 'steady state'. It is possible to observe events in the pre-steady state period using specialized apparatus; this can give additional information on the rates of individual steps in the overall reaction (see Chapter 9, section 9.9.4).

4.3.4 Inhibition of enzyme-catalysed reactions

Although a full discussion of the inhibition of enzyme-catalysed reactions is beyond the scope of this book, it is important that you are able to analyse appropriate data to determine the type of inhibition being observed in any particular case and to evaluate the strength of the interaction between enzyme and inhibitor in terms of a suitable dissociation constant. We will confine our attention to those inhibitors which bind reversibly to enzymes.

In the mechanism quoted previously:

$$E + S \rightleftharpoons ES \rightarrow E + P$$

we could envisage a general scheme for the way in which an inhibitor (I) might interact with E and the ES complex.

We can consider three limiting cases for enzyme–inhibitor interaction:

A. *Competitive inhibition* The inhibitor (I) binds to E but not to ES (i.e. $K_{ESI} = \infty$ which means that the ESI complex has no tendency to form). In this case, the addition of I will pull some of E over to the EI complex. However, addition of increasing concentrations of S will eventually overcome the effect of I, since the balance will progressively swing towards formation of ES rather than EI. This is termed competitive inhibition; V_{max} remains unchanged, but K_m is

raised (reflecting the higher [S] to be added to overcome the effects of I). The most likely explanation for competitive inhibition is that although the inhibitor cannot undergo the reaction, it has a structural resemblance to the substrate and therefore binds at the active site, preventing the binding of S.

In quantitative terms, K_m in the presence of inhibitor is raised by a factor $\left(1 + \frac{[I]}{K_{EI}}\right)$ compared with the absence of inhibitor (K_{EI} is the dissociation constant of the EI complex). V_{max} remains unchanged.

Competitive inhibitors are widely studied to define the way that the substrate and enzyme interact, and as the basis for designing potential therapeutic drugs. Examples of the latter include angiotensin-converting enzyme inhibitors to control hypertension, and inhibitors of hydroxymethylglutaryl coenzyme A reductase (HMG CoA reductase) to lower blood cholesterol levels and thereby decrease the risk of heart attacks.

B. *Non-competitive inhibition*: The inhibitor (I) binds to E and ES with equal affinity (i.e. $K_{EI} = K_{ESI}$). In this case, both E and ES will be pulled over to the complexes EI and ESI, respectively, and the net effect will be that a certain fraction of the enzyme is complexed with I and unable to catalyse the reaction. The remaining enzyme will have the same K_m for substrate as in the absence of inhibitor. This is termed non-competitive inhibition. The inhibitor cannot bind to the active site, since it does not affect the affinity of the enzyme for substrate.

Effectively a proportion of the enzyme has been removed from the system; this could also be achieved by irreversible inhibition of part of the enzyme present. The case discussed here is more properly termed pure non-competitive inhibition. There is in fact a range of possible effects of inhibitors on V_{max} and K_m, which are too complex to be dealt with in detail here.

In quantitative terms, V_{max} in the presence of inhibitor is lowered by a factor $\left(1 + \frac{[I]}{K_{EI}}\right)$ compared with the absence of inhibitor. K_m remains unchanged.

C. *Uncompetitive inhibition*: The inhibitor binds only to the ES complex and not to E ($K_{EI} = \infty$, i.e. there is no tendency for the EI complex to form). In the presence of I, E will be pulled over to the ES complex, which will then be pulled towards the ESI complex. This will have the effect of lowering both K_m (because E is pulled over to ES) and V_{max} (because some of the enzyme in the form of ESI will be unable to take part in the catalytic reaction). This is termed uncompetitive inhibition and is extremely rare in one-substrate reactions (although several examples are found in multi-substrate reactions). The inhibition could arise from a structural change occurring on binding of substrate to enzyme which then creates a binding site for the inhibitor.

In quantitative terms, both K_m and V_{max} are lowered in the presence of inhibitor by a factor $\left(1 + \frac{[I]}{K_{ESI}}\right)$ compared with the absence of inhibitor (K_{ESI} is the dissociation constant of the ESI complex to give ES + I).

The three different types of inhibitors can be easily recognized by their effects on the kinetic plots as described in section 4.4.

? SELF TEST

Check that you have mastered the key concepts at the start of the section by attempting the following questions.

ST 4.7 A certain drug binds to a target receptor with a K_d of 5×10^{-8} M: what is the ΔG^0 for dissociation of this complex at 37°C?

ST 4.8 For an enzyme-catalysed reaction which obeys Michaelis–Menten (hyperbolic) kinetics, what fold change in [*S*] is required to go from a rate of 20% V_{max} to 80% V_{max}?

ST 4.9 For a binding process which obeys the Hill equation (eqn. 4.25), assume that $K = 1$ mM and that [*L*] changes from 0.5 to 2 mM. What change in fractional saturation occurs over this range for the cases (a) when $h = 2.0$, and (b) when $h = 3.0$?

Answers

ST 4.7 The ΔG^0 for the dissociation = 43.3 kJ mol^{-1}.

ST 4.8 A 16-fold change is required.

ST 4.9 The changes in fractional saturation are: (a) from 0.2 to 0.8; (b) from 0.11 to 0.89. These show the much greater sensitivity to changes in [*L*] compared with hyperbolic binding (eqn. 4.22), where the change is only 2-fold, from 0.33 to 0.67.

Attempt Problem 4.7 at the end of the chapter.

4.4 Analysis of binding and kinetic data

KEY CONCEPTS

- Understanding the graphical procedures used to analyse binding and kinetic data
- Expressing the parameters obtained in the correct units and checking that they are reasonable in terms of the data supplied

As we have seen for systems where there are no interactions between the binding sites, there are essentially two parameters to be determined from the experimental data, one representing the property of the fully saturated protein (V_{max} or n), and the other representing the concentration of ligand or substrate to achieve half-saturation (K_m or K_d). In order to obtain these parameters experimentally we need to make measurements of the extent of binding or the enzyme activity at different concentrations of ligand or substrate. From these data, it is usually difficult to estimate the limiting value (V_{max} or n) directly since this would involve the use of very high concentrations of ligand or substrate.

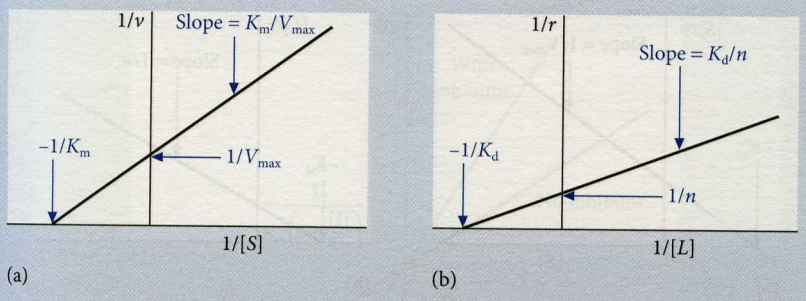


Fig. 4.13 Analysis of enzyme kinetic data (a) and ligand-binding data (b). The plots are known as Lineweaver–Burk and Hughes–Klotz plots, respectively.

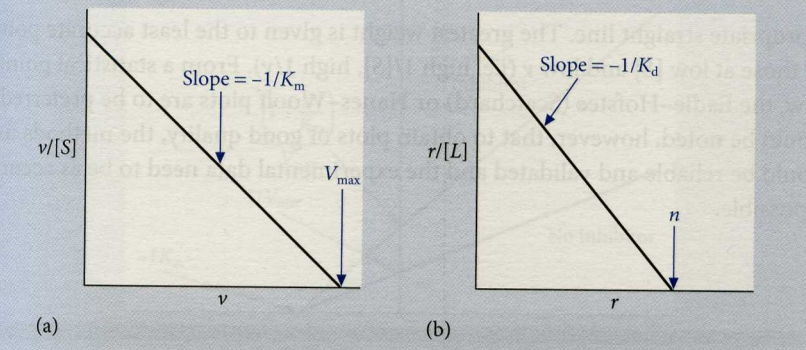


Fig. 4.14 Analysis of enzyme kinetic data (a) and ligand-binding data (b). The plots are known as Eadie–Hofstee and Scatchard plots, respectively.

We can use either direct, computer-based, fitting to the saturation curve (non-linear regression; see Chapter 2, section 2.6.5) or transformations (see Chapter 2, section 2.5.2) of the saturation equations (eqns. 4.22, 4.23, and 4.26) to give linear plots, which are termed Lineweaver–Burk (Hughes–Klotz), Eadie–Hofstee (Scatchard) and Hanes–Woolf. (The names in brackets are used for the plots used to analyse binding, rather than enzyme kinetic data). From the slopes and intercepts of these plots (Figs. 4.13, 4.14, and 4.15) the parameters (V_{max} or n; K_m or K_d) can be obtained. Figure 4.16 depicts the effects of the various types of inhibitors (section 4.3.4) on the Lineweaver–Burk plots.

The Lineweaver–Burk plot is the most commonly used for analysis of enzyme kinetics; it involves plotting the two variables, or rather derivatives of them, on separate axes. However, because of the reciprocal nature of the axes, the error distribution is highly non-uniform, which makes it difficult to decide on the most

The criteria for assessing the results of application of non-linear regression to binding or kinetic data are described in Chapter 2, section 2.6.5.

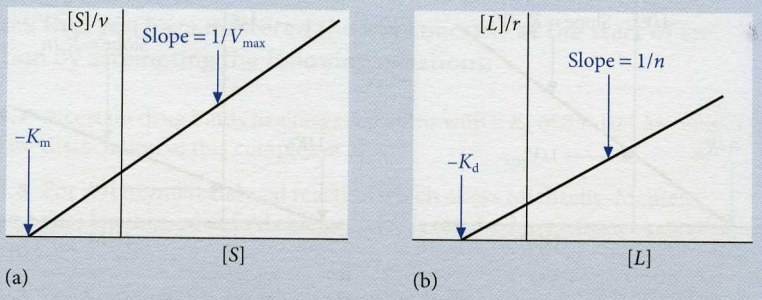


Fig. 4.15 Analysis of enzyme kinetic data (a) and ligand-binding data (b). The plots are known as Hanes–Woolf plots.

appropriate straight line. The greatest weight is given to the least accurate points, i.e. those at low [*S*] and low v (i.e. high 1/[*S*], high 1/v). From a statistical point of view, the Eadie–Hofstee (Scatchard) or Hanes–Woolf plots are to be preferred. It should be noted, however, that to obtain plots of good quality, the methods used should be reliable and validated and the experimental data need to be as accurate as possible.

WORKED EXAMPLE

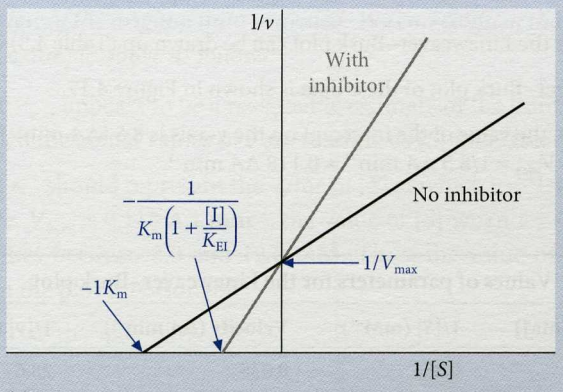
Analyse the data in Table 4.4 showing the variation of rate with substrate concentration for an enzyme-catalysed reaction, using the Lineweaver–Burk plot. State the values of K_m and V_{max} you obtain, quoting the correct units.

Table 4.4 Variation of velocity of an enzyme-catalysed reaction with substrate concentration

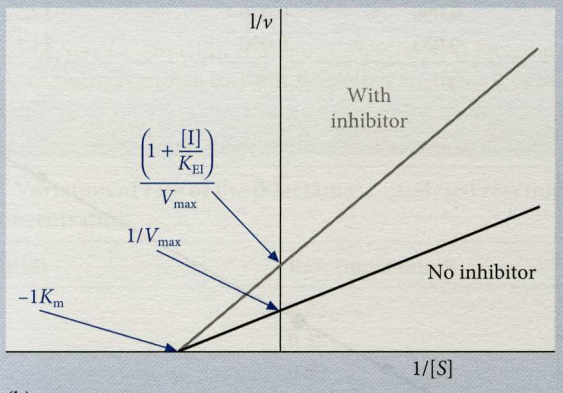
[Substrate] (mM)	Velocity (ΔA min^{-1})
4.0	0.035
8.0	0.054
12.0	0.066
20.0	0.080
30.0	0.090

STRATEGY

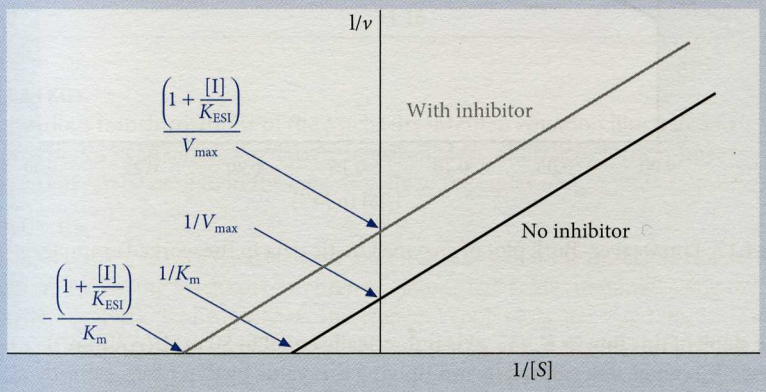
The data are manipulated to be in the form required for the Lineweaver–Burk plot (1/v vs. 1/[*S*]). It is important to choose appropriate scales for the plot so that the intercepts and slope can be read off easily and accurately.



(a)



(b)



(c)

Fig. 4.16 The effects of different types of inhibitor on Lineweaver–Burk plots of enzyme kinetic data. (a), (b), and (c) show competitive, non-competitive, and uncompetitive inhibition, respectively.

SOLUTION

The values for the Lineweaver–Burk plot can be drawn up (Table 4.5):

The Lineweaver–Burk plot of these data is shown in Figure 4.17.

From the plot, the value of the intercept on the y-axis is 8.5 $(\Delta A\ \text{min}^{-1})^{-1}$. This is $1/V_{max}$, hence $V_{max} = 1/8.5\ \Delta A\ \text{min}^{-1} = 0.118\ \Delta A\ \text{min}^{-1}$.

Table 4.5 Values of parameters for the Lineweaver–Burk plot

[Substrate] (mM)	$1/[S]$ (mM^{-1})	Velocity ($\Delta A\ \text{min}^{-1}$)	$1/[v]$ $(\Delta A\ \text{min}^{-1})^{-1}$
4.0	0.25	0.035	28.6
8.0	0.125	0.054	18.5
12.0	0.083	0.066	15.2
20.0	0.050	0.080	12.5
30.0	0.033	0.090	11.1

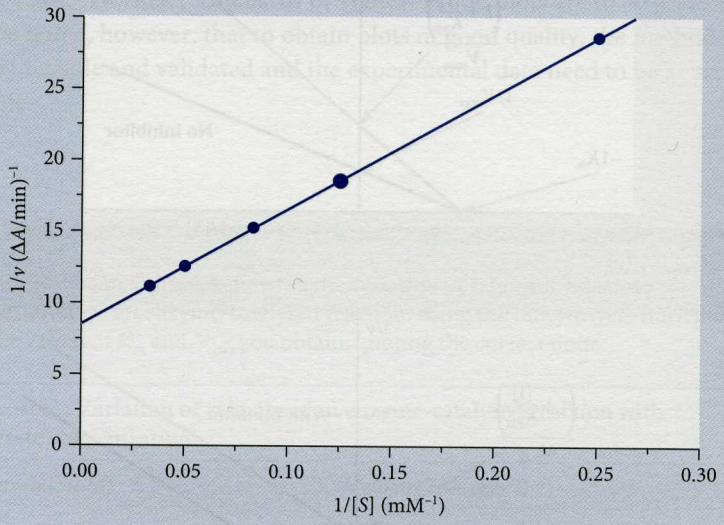


Fig 4.17 Lineweaver–Burk plot of enzyme kinetic data in the worked example.

The slope of this line ($= K_m/V_{max}$) is numerically equal to 80 mM/($\Delta A\ \text{min}^{-1}$). Hence K_m can be determined by multiplying this value by that of V_{max} already determined; i.e. $K_m = 80 \times 0.118\ \text{mM} = 9.44\ \text{mM}$. Alternatively, K_m can be determined from the extrapolated intercept on the x-axis, which is equal to $-1/K_m$. (Note that if the straight line is extended (extrapolated) beyond the $1/v$ axis as shown, the intercept is negative because the values of $1/[S]$ on this side of the origin are negative.) This allows the value of K_m to be calculated directly (in this case the value of the intercept is $-0.106\ \text{mM}^{-1}$, hence $K_m = 1/0.106\ \text{mM} = 9.44\ \text{mM}$.

It is always good practice to assess whether your values of V_{max} *and* K_m *are reasonable or not in view of the original data provided. We can confirm that this is the case for the worked example above as follows:*

- The value of V_{max} appears to be a reasonable estimate of the limiting value towards which the actual values of v are tending as [S] increases
- The value of K_m should represent the value of [S] at which $v = {}^1/_2 V_{max}$. In the present example, V_{max} is 0.118 ΔA min^{-1}, the value of [S] when $v = 0.059$ ΔA min^{-1} (i.e. ${}^1/_2 V_{max}$) lies between 8.0 and 12.0 mM. Thus, the value of K_m (9.44 mM) derived from the plot is reasonable.

WORKED EXAMPLE

The rate of hydrolysis of a penicillin analogue catalysed by β-lactamase varied with substrate concentration as shown in Table 4.6. What are the values of K_m and V_{max} for this reaction?

Table 4.6 Variation of rate of the β-lactamase-catalysed reaction with substrate concentration

[Substrate] (μM)	Rate (nmol min^{-1})
1	0.22
2	0.38
3	0.50
5	0.68
10	0.90
30	1.16

STRATEGY

The various transformations of the Michaelis–Menten equation illustrated in Figs. 4.13–4.15 can be used to derive the parameters; alternatively, non-linear regression can be used to fit the data directly to the equation.

SOLUTION

All three plots give very good straight lines indicating that the experimental data conform well to the Michaelis–Menten equation. The values obtained using the Lineweaver–Burk, Eadie–Hofstee, and Hanes–Woolf plots are: V_{max} 1.36, 1.37, and 1.36 nmol min^{-1}, respectively, and K_m 5.26, 5.18, and 5.12 μM, respectively. The non-linear regression fitting gives $V_{max} = 1.36$ (standard error 0.01) nmol min^{-1} and $K_m = 5.10$ (standard error 0.06) μM. The very low standard errors (approximately 1% of the actual values of the parameters) confirm the goodness of the fit of the experimental data to the to the Michaelis–Menten equation. The values of the parameters can be seen to be reasonable by the criteria given above. An extension of this problem is given in the self-test exercise **ST 4.10** at the end of this section.

WORKED EXAMPLE

The guanidinium ion is known to act as an inhibitor of the trypsin-catalysed hydrolysis of a model substrate, *N*-benzoyl-L-arginine-4-nitroanilide (BAPNA). Analyse the data in Table 4.7 to determine the type of inhibition being shown and evaluate the appropriate dissociation constant for the enzyme–guanidinium complex.

STRATEGY

The Lineweaver–Burk plots for the data obtained in the presence and absence of inhibitor will indicate the type of inhibition observed (see Fig. 4.16).

Table 4.7 Inhibition of the trypsin-catalysed hydrolysis of BAPNA by guanidinium ions

[BAPNA] (mM)	Velocity (μmol min^{-1} mg^{-1})	
	−guanidinium	+guanidinium (3 mM)
0.3	3.8	2.0
0.6	5.8	3.5
1.0	7.2	4.8
1.5	8.2	5.9
2.0	8.9	6.8
2.5	9.3	7.4

SOLUTION

From any of the appropriate plots (e.g. Lineweaver–Burk) it can be seen that in the presence of guanidinium the V_{max} remains constant (11.5 μmol min^{-1} mg^{-1}), but that K_m is increased (from 0.6 to 1.4 mM). Thus, guanidinium acts as a competitive inhibitor; it should be noted that the arginine side chain of the substrate contains the guanidinium group. There is a 2.3-fold increase in K_m, so $(1 + ([I]/K_{EI})) = 2.3$. Putting $[I] = 3$ mM, this gives $K_{EI} = 2.3$ mM.

The action of proteases on nitroanilide substrates is described in Chapter 9, section 9.9.4. The structures of arginine and the guanidinium ion are given in Chapter 1, section 1.8.

WORKED EXAMPLE

The data in Table 4.8 were obtained for the binding of NAD^+ to the enzyme malate dehydrogenase (15 μM), which consists of two polypeptide chains. Analyse the data using the Scatchard equation (see Chapter 2, section 2.5.2) to obtain the number of binding sites and the K_d for the interaction.

Table 4.8 Binding of NAD^+ to malate dehydrogenase

$[NAD^+]_{free}$ (μM)	r
5	0.33
10	0.57
20	0.89
30	1.09
50	1.33
75	1.50
100	1.60

STRATEGY

The data must be put into the correct form for the Scatchard plot ($r/[NAD^+]_{free}$ vs r (Table 4.9). It is important to make sure that the correct units are noted.

Table 4.9 Values of parameters for the Scatchard plot

$[NAD^+]_{free}$ (μM)	r	$r/[NAD^+]_{free}$ $(\mu M)^{-1}$
5	0.33	0.067
10	0.57	0.057
20	0.89	0.045
30	1.09	0.036
50	1.33	0.027
75	1.50	0.020
100	1.60	0.016

SOLUTION

The Scatchard plot is shown in Fig. 4.18. The data points fall on a straight line of slope $-0.04\ \mu M^{-1}$. Thus $K_d = -(-1/0.04)\ \mu M = 25\ \mu M$. The intercept on the x-axis is equal to the n, the number of binding sites. There are thus two binding sites for NAD^+ on malate dehydrogenase, i.e. one per polypeptide chain. Since the plot is linear, these sites are equivalent and independent.

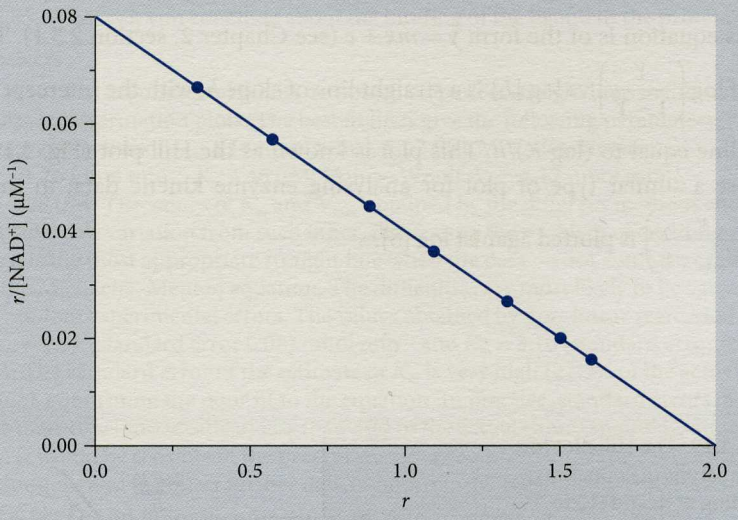


Fig. 4.18 Scatchard plot analysis of data for the binding of NAD^+ to the enzyme malate dehydrogenase in the worked example.

Note that it is essential to express the quantities derived from of the analysis of kinetic or binding data in the correct units. The units of K_m or K_d are those of concentration (M, mM, μM, etc. or in the case of a species of unknown molecular mass this might be expressed as % (w/v)). The units of V_{max} are those in which v is expressed (e.g. μmol min^{-1} or $\Delta A\ s^{-1}$). The parameter r in the binding equation does not have units as it represents a ratio. The value of r when there are multiple binding sites should be correlated with other data such as the number of subunits in a multi-subunit protein (e.g. four binding sites for NADH in lactate dehydrogenase, a tetrameric protein).

If a ligand binds to multiple sites on a protein in a cooperative fashion, the binding can be conveniently analysed by the Hill equation (eqn. 4.25):

$$Y = \frac{[L]^h}{K + [L]^h} \qquad 4.25$$

Rearranging and taking logarithms of both sides, we obtain:

$$\log\left(\frac{Y}{1-Y}\right) = h \log [L] - \log K \qquad 4.27$$

This equation is of the form $y = mx + c$ (see Chapter 2, section 2.5.1). Thus, a plot of $\log\left(\frac{Y}{1-Y}\right)$ vs $\log [L]$ is a straight line of slope h, with the intercept on the $y = 0$ line equal to $(\log K)/h$. This plot is known as the Hill plot (Fig. 4.19). We can use a similar type of plot for analysing enzyme kinetic data; in this case $\log\left(\frac{v}{V_{max} - v}\right)$ is plotted against $\log [S]$.

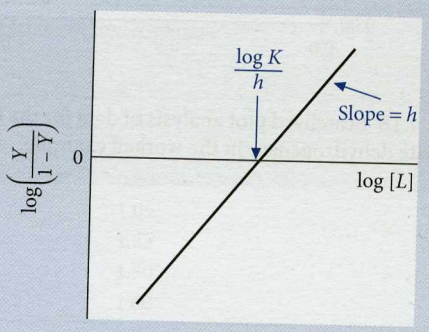


Fig. 4.19 The Hill plot for the analysis of cooperative binding of ligand (L) to a protein according to eqn. 4.27. From the slope of the line (h) and the value of $\log [L]$ at half saturation (i.e. when $Y = 0.5$ and hence $\log\left(\frac{Y}{1-Y}\right) = 0$), the value of K can be evaluated as shown.

SELF TEST ?

Check that you have mastered the key concepts at the start of the section by attempting the following questions.

ST 4.10 In a further experiment on the hydrolysis of the pencillin analogue catalysed by β-lactamase the following data were obtained. What are the values of K_m and V_{max} for the reaction?

[Substrate] (μM)	Rate (nmol min^{-1})
1	0.24
2	0.34
3	0.56
5	0.76
10	0.90
30	0.95

ST 4.11 Addition of an inhibitor (80 μM) led to no change in the V_{max} of an enzyme-catalysed reaction, but the K_m for the substrate was raised from 22 to 68 μM. What can you conclude about the interaction of the inhibitor with the enzyme?

ST 4.12 A Scatchard plot was used to analyse the binding of a ligand to a protein with four polypeptide chains. The term $r/[L]_{free}$ was plotted on the y-axis and r on the x-axis (r is the mol ligand bound per mol enzyme and $[L]_{free}$ is the concentration of free ligand). The plot showed a straight line of negative slope, with an intercept on the y-axis (i.e. when $x = 0$) of 0.058 μM^{-1}; the intercept on the x-axis (i.e. when $y = 0$) was 3.85. What can you conclude about the binding of the ligand to the protein?

Answers

ST 4.10 The experimental points do not fall on perfect straight lines in the various linear transformation plots. The best fit lines give the following parameters: Lineweaver–Burk, V_{max} 1.14 nmol min^{-1}, K_m 3.58 μM; Eadie–Hofstee, V_{max} 1.26 nmol min^{-1}, K_m 4.46 μM; Hanes–Woolf, V_{max} 1.12 nmol min^{-1}, K_m 3.29 μM. The values of K_m and V_{max} obtained by the different methods show considerable variation from each other, illustrating the problem of deciding what is the most appropriate straight line when the data do not conform exactly to the Michaelis–Menten equation. The difficulties are most likely to be caused by random experimental errors. The values obtained by non-linear regression are $V_{max} = 1.11$ (standard error 0.08) nmol min^{-1} and $K_m = 3.16$ (standard error 0.68) μM. The standard error of the estimate of K_m is very high (21.5% of the actual value), confirming the poor fit to the equation. In practice, standard errors of less than 10% (and ideally of less than 5%) of the actual values should be aimed for. It would be sensible in the present case to attempt to refine the experimental techniques and to collect further data to improve the reliability of the estimates.

ST 4.11 The inhibitor is competitive, from the change in K_m, K_{EI} can be calculated to be 38 μM.

ST 4.12 There are 3.85 binding sites per enzyme, i.e. very close to one per polypeptide chain; these are equivalent and independent. The slope of the Scatchard plot $= -\frac{0.058}{3.85}$ μM^{-1}, i.e. −0.0151 μM^{-1}. This is equal to $-\frac{1}{K_d}$, so that $K_d = 66$ μM.

Attempt Problems 4.8–4.11 at the end of the chapter.

4.5 Calculation of k_{cat} and k_{cat}/K_m for an enzyme-catalysed reaction

KEY CONCEPTS

- Calculating k_{cat} for an enzyme-catalysed reaction given appropriate data
- Calculating the ratio k_{cat}/K_m for an enzyme-catalysed reaction and understanding its importance as a measure of the efficiency of enzyme action

The parameter k_{cat} is a rate constant which denotes how rapidly (under saturating conditions), one molecule of enzyme can convert one molecule of substrate to product. In order to calculate k_{cat}, it is necessary to know V_{max} in terms of the number of moles substrate converted per unit time and the amount of enzyme present in terms of the number of moles. (The latter is calculated from the mass of enzyme used in the assay and the molecular mass of the enzyme.) The calculation of k_{cat} is illustrated in the worked example below.

We can, of course, also calculate k_{cat} if we know the molar concentration of substrate converted per unit time and the molar concentration of enzyme present.

✓ WORKED EXAMPLE

Dehydroquinase catalyses the conversion of 3-dehydroquinate to 3-dehydroshikimate and water. The V_{max} for the reaction catalysed by the type II enzyme from *Streptomyces coelicolor* is 0.011 μmol converted per min when 0.025-μg enzyme is added. The molecular mass of each subunit (i.e. each active site) is 16.5 kDa. Calculate the value of k_{cat} for this enzyme.

STRATEGY

This type of calculation involves a number of steps. It is important to set these out (with explanations) and to make sure that you keep track of the units.

SOLUTION

The V_{max} is 0.011 μmol min^{-1}, i.e. 0.011/60 μmol s^{-1} = 1.83×10^{-10} mol s^{-1} (183 pmol s^{-1}). The amount of enzyme present is 0.025 μg, which is equivalent to $0.025 \times 10^{-6}/16\,500$ mol = 1.52×10^{-12} mol (1.52 pmol). (Note 1 mol enzyme is 16 500 g.)Hence, k_{cat} = 183/1.52 pmol $pmol^{-1}$ s^{-1} = 120 mol mol^{-1} s^{-1} (this could also be expressed as 120 s^{-1}). At a molecular level, the value of k_{cat} means that every 8.3 ms, a molecule of substrate (3-dehydroquinate) diffuses to and binds to the enzyme, is converted to products (3-dehydroshikimate and water) which then dissociate from the enzyme.

Note the importance in the calculation of k_{cat} of specifying the correct molecular mass. In this case, the enzyme consists of 12 polypeptide chains (subunits) each of molecular mass 16.5 kDa. The value of k_{cat} obtained refers to the events at the catalytic site on each subunit.

The values of k_{cat} generally range from about 10 s^{-1} for enzymes catalysing complex biosynthetic reactions to about 10^6 s^{-1} for enzymes such as catalase or carbonic anhydrase which catalyse chemically simple reactions (the breakdown of hydrogen peroxide to give water and oxygen, and the hydration of CO_2 to give carbonic acid, respectively).

The ratio k_{cat}/K_m is a useful parameter to characterize an enzyme. It can indicate the specificity of an enzyme towards a given substrate in cases where an enzyme can act on a number of possible substrates. The absolute value of the ratio can also

indicate the catalytic efficiency of an enzyme. In the case of dehydroquinase in the worked example, the K_m for 3-dehydroquinate is 105 μM, so that the value of $k_{cat}/K_m = 1.14 \times 10^6\ M^{-1}\ s^{-1}$. However, for a number of enzymes, such as triosephosphate isomerase, fumarase and acetylcholinesterase, the value of k_{cat}/K_m is about $10^8\ M^{-1}\ s^{-1}$, which effectively represents the diffusion limit, i.e. the rate of the overall reaction is limited by the rate at which substrate can encounter the active site of the enzyme, with all subsequent steps (the chemical reaction, dissociation of products, etc.) occurring more rapidly. This phenomenon is sometimes referred as 'catalytic perfection'.

Although an enzyme might have achieved 'perfection' in terms of its catalytic efficiency, it may possibly still be 'improved' in terms of regulatory properties or stability in different physiological situations.

SELF TEST ?

Check that you have mastered the key concepts at the start of the section by attempting the following questions.

ST 4.13 For the reaction catalysed by fumarase (fumarate ⇌ malate + H_2O), the K_m for fumarate is 4.5 μM and the V_{max} is 45 000 mol fumarate mol^{-1} enzyme min^{-1}. Calculate k_{cat} (in units of s^{-1}) and the ratio k_{cat}/K_m (in units of $M^{-1}\ s^{-1}$) and comment on the value of this ratio.

ST 4.14 The enzyme β-lactamase catalyses the hydrolysis of penicillin and a number of its derivatives. In a 1-mL reaction volume to which 0.015 μg enzyme was added, the V_{max} was found to be 10.2 μM substrate hydrolysed per minute and the K_m was 5.2 μM. The molecular mass of the enzyme is 29.6 kDa. Calculate k_{cat} and k_{cat}/K_m for this enzyme-catalysed reaction.

Answers

ST 4.13 The values of k_{cat} and k_{cat}/K_m are $750\ s^{-1}$ and $1.67 \times 10^8\ M^{-1}\ s^{-1}$, respectively; the latter value is close to the diffusion-controlled limit, i.e. to 'catalytic perfection'.

ST 4.14 The values of k_{cat} and k_{cat}/K_m are $335\ s^{-1}$ and $6.44 \times 10^7\ M^{-1}\ s^{-1}$, respectively.

Attempt Problems 4.12 and 4.13 at the end of the chapter.

4.6 Radioactivity

KEY CONCEPTS

- Knowing the three types of radiation emitted by unstable nuclei
- Knowing the definitions of the units of radioactivity (Curie and Becquerel)
- Understanding the terms efficiency of counting and specific radioactivity and how they are used in calculations

The nucleus of an unstable isotope will decay to produce one of the following types of radiation, namely α (corresponding to He nuclei), β (corresponding to

Table 4.10 Properties of the radioactive isotopes most commonly used in studies of proteins

Isotope	Half-life	Maximum energy (eV)*
^{3}H	12.3 years	1.9×10^{4}
^{14}C	5730 years	1.56×10^{5}
^{31}P	14.3 days	1.71×10^{6}
^{35}S	87.4 days	1.67×10^{5}

*The electrons emitted will have a range of energies; the values shown are the maximum energies observed in each case. 1 eV (electron volt) is equivalent to 96.5 kJ mol^{-1}.

electrons), and γ (corresponding to high-energy photons). In some cases, more than one type will be produced e.g. ^{131}I decays giving both β- and γ-rays.

The much higher energy of the electrons emitted by ^{32}P means that it is very important to adopt stringent safety procedures when working with this isotope.

The principal isotopes used in biochemical research are β-emitters, although γ-emitting isotopes of iodine have a number of specialized applications, particularly in immunology. Incorporation of a radioactive isotope provides a very sensitive means of tracking a particular compound, especially in studies of enzyme-catalysed reactions or metabolism, and in measuring its binding to another molecule. Since radioactivity arises from the decay of individual nuclei, it is possible to measure very small amounts of a radioactive compound (in the pmol or fmol range). The isotopes most commonly employed in studies of proteins are ^{3}H, ^{14}C, ^{32}P, and ^{35}S, the properties of which are listed in Table 4.10. (There are no convenient radioactive isotopes of N or O available.)

The Curie is named in honour of Pierre and Marie Curie, who worked in France on the identification and purification of radioactive elements (principally polonium and radium). Marie was Polish by birth (Maria Sklodowska). The Becquerel is named in honour of Henri Becquerel, who discovered the phenomenon of radioactivity in a salt of uranium.

The amount of radioactivity in a substance is quoted in two types of units. The older, but still used, unit is the Curie (Ci), which is defined as 3.7×10^{10} disintegrations per second (dps). This corresponds to the activity of 1g pure radium. Because the Curie is such a large amount of radioactivity, we typically use much smaller quantities (mCi or μCi) in experiments. The other unit is the Becquerel (Bq), which corresponds to 1 dps; this is, conversely, such a small amount of radioactivity that we would generally be using kBq or MBq in experiments.

A radioactive compound will be supplied with a stated specific radioactivity, e.g. ^{3}H-alanine might be supplied at 50 Ci mol^{-1} (this would correspond to 1850 GBq mol^{-1}). For experimental purposes, this would usually be mixed with a large excess of unlabelled alanine to give a specific radioactivity of, for example 0.5 Ci mol^{-1}. *Knowledge of the specific radioactivity of a sample containing a radioactive compound allows us to convert the observed radioactivity into the amount (mol) of that compound present.*

Radioactivity is usually measured by liquid scintillation counting. The sample is dissolved or suspended in a suitable solvent containing one or more fluorescent compounds (known as fluors). Emission of an electron by the radioactive nucleus will excite the fluor (this can occur via excitation of a solvent molecule). Emission of radiation (scintillations) by the fluor can be detected by a photomultiplier. The efficiency of detection of the radioactive decay depends on a number of factors including the physical state of the radioactive material and the energy of the

electrons emitted. In the case of ^{3}H-labelled compounds the efficiency is fairly low (typically 20–30%), but is much higher for ^{14}C-labelled, and especially for ^{32}P-labelled compounds. *We must measure the efficiency of counting to be able to convert the observed counts per second (cps) to disintegrations per second (dps)*

Radioactivity is a very sensitive tool because events could, in theory at least, be detected at very nearly the scale of individual atoms and molecules. This makes it ideally suited to the measurements of very small amounts of material such as may occur in biological systems. In addition, the introduction of radioactive isotopes does not affect the chemical behaviour of atoms significantly (although there can be effects on the rates of some reactions), so that they serve as ideal probes and tracers for molecules in complex systems. In this book, we shall confine our attention to the use of radioactive isotopes for assays of enzyme activity and for the monitoring of binding processes.

In practice, radioactivity should only be used as a probe with a small proportion of the relevant molecules containing the radioactive isotope. When an isotope decays it can generate a new element with different chemical characteristics, which may lead to damage to the molecule concerned. Thus, ^{32}P and ^{35}S decay by β-emission to generate ^{32}S and ^{35}Cl, respectively. The radioactive compound supplied would generally be mixed with a large molar excess of the non-radioactive compound to obtain the working solution.

WORKED EXAMPLE ✓

A sample of ATP labelled in the γ-phosphate position with ^{32}P was purchased and mixed with a solution of unlabelled ATP to give a specific radioactivity of 5 Ci mol^{-1}; this material was then stored in the freezer. After 43 days an experiment was carried out in which this solution was used to monitor the incorporation of phosphate into a protein of molecular mass 60 kDa. After 1 mL of the protein solution (1.3 mg mL^{-1}) had been incubated with the radioactive ATP and necessary factors for reaction, the mixture was treated with trichloroacetic acid to stop the reaction and precipitate the protein. The radioactivity incorporated was 25 200 counts per minute (cpm), and the efficiency of counting was 90%. What was the extent of incorporation of phosphate into the protein?

STRATEGY

Given the relatively short half-life of the ^{32}P isotope, it is necessary to calculate the specific radioactivity on the day of the experiment. Calculation of the extent of incorporation requires knowledge of the amounts (numbers of moles) of protein and of radioactivity incorporated. The working should be clearly set out and explained.

SOLUTION

The ATP sample was used 43 days after purchase; reference to Table 3.1 shows that this time corresponds to 3 half-lives of the ^{32}P isotope. Thus, on the day of the experiment, the specific radioactivity will be $0.5 \times 0.5 \times 0.5$ (i.e. 0.125) times the original, namely 0.625 Ci mol^{-1}, or $0.625 \times 3.7 \times 10^{10}$ dps $mol^{-1} = 2.313 \times 10^{10}$ dps mol^{-1}. The radioactivity incorporated was 25 200 cpm or 25 200/60 cps = 420 cps. Since the efficiency was 90%, this would correspond to $420 \times 100/90$ dps = 467 dps Using the value for the specific radioactivity we can calculate that this would correspond to $467/(2.313 \times 10^{10})$ mol = 20.2 nmol phosphate incorporated. The amount of protein taken was 1.2 mg; this corresponds to $1.2 \times 10^{-3}/60\,000$ mol protein = 20 nmol protein. Thus, the incorporation of phosphate amounts to 20.2/20 = 1.01 mol mol^{-1} protein.

WORKED EXAMPLE

The half-life of ^{14}C is 5730 years. What is the maximum specific radioactivity (Bq mol^{-1}) of a sample of [^{14}C] glucose, which has the molecular formula $C_6H_{12}O_6$? (Avogadro's number is 6.02×10^{23} mol^{-1}.)

STRATEGY

From the half-life, the rate constant can be calculated. When multiplied by Avogadro's number, this will give the initial rate of radioactive decay for 1 mol of ^{14}C atoms; this value has to be multiplied by 6 to obtain the rate for 1 mol glucose, which has 6 carbon atoms.

SOLUTION

The $t_{1/2}$ (5730 years) corresponds to 1.81×10^{11} s; thus the rate constant $= 0.693/(1.81 \times 10^{11})$ s^{-1}, i.e. 3.835×10^{-12} s^{-1}. The initial rate of decay for 1mol ^{14}C atoms $= 3.835 \times 10^{-12} \times 6.02 \times 10^{23}$ dps (Bq) $= 2.31 \times 10^{12}$ Bq. For glucose, the rate would be 1.39×10^{13} Bq mol^{-1} or 13.9 TBq mol^{-1} (T = tera = 10^{12}).

SELF TEST

Check that you have mastered the key concepts at the start of the section by attempting the following questions.

ST 4.15 The radioactivity in a sample of [^{3}H] leucine was measured as 8650 cpm; the efficiency of counting was 22%. The specific radioactivity of the sample was 1.43 GBq mol^{-1}. Calculate the dps of the sample and the number of moles of leucine present.

ST 4.16 What is the maximum radioactivity of 1 mol of ^{32}P atoms, given that the half-life is 14.3 days and Avogadro's number is 6.02×10^{23} mol^{-1}.

Answers

ST 4.15 There are 655 dps in the sample and hence 4.58×10^{-7} mol, i.e. 0.458 µmol present.

ST 4.16 The maximum radioactivity is 3.77×10^{17} Bq mol^{-1}.

Attempt Problems 4.14–4.16 at the end of the chapter.

4.7 Problems

Full solutions to odd-numbered problems are available to all in the student section of the Online Resource Centre at www.oxfordtextbooks.co.uk/orc/price/. Full solutions to even-numbered problems are available to lecturers only in the lecturer section of the Online Resource Centre.

4.1 For the formation of the complex between Mg^{2+} ions and the chelating agent $EDTA^{4-}$ at 20°C, $\Delta G^0 = -48.7$ kJ mol^{-1} and $\Delta H^0 = 13.1$ kJ mol^{-1}. Calculate the ΔS^0 for the formation of the $MgEDTA^{2-}$ complex, and comment on your answer.

4.2 For the hydrolysis of ATP ($ATP + H_2O \rightleftharpoons ADP + P_i$), $\Delta G'^0_{310} = -31.0$ kJ mol^{-1}. In resting muscle at 310 K, the concentrations of ATP, ADP and P_i are 10, 3, and 1 mM, respectively. Is the ATP hydrolysis reaction at equilibrium in resting muscle? If not, what is the value of ΔG under these conditions?

4.3 The average daily energy requirements of an adult male human are 10 000 kJ.

Using the value obtained in Problem 4.2 for the ΔG corresponding to ATP hydrolysis and synthesis under cellular conditions, calculate the mass of ATP turned over per day. Assume that 1 mol of ATP corresponds to 550 g.

4.4 Glycogen phosphorylase catalyses the following reaction in the mobilization of glycogen reserves:

$$(\text{glycogen})_n + P_i \rightleftharpoons (\text{glycogen})_{n-1} + \text{glucose-1-phosphate}$$

for which $\Delta G'^0 = 3.05$ kJ mol^{-1} at 37°C. Assuming that the concentrations of P_i and glucose-1-phosphate are equal, does the equilibrium lie in favour of glycogen synthesis or degradation at 310 K? In muscle the concentrations of P_i and glucose-1-phosphate are 10 mM and 30 μM, respectively. Does glycogen phosphorylase catalyse the net synthesis or degradation of glycogen in muscle?

4.5 The radioactivity of a sample of ATP labelled at the γ-phosphate with the isotope ^{32}P was measured at different times with the following results.

Time (days)	0	5	10	20	30	50
Radioactivity (cpm)	53 450	41 950	32 920	20 270	12 490	4740

Show that the decay of the isotope is a first-order process and determine the rate constant and half-life for this process.

4.6 When the enzyme creatine kinase is incubated with iodoacetamide, there is a complete loss of activity due to reaction of a single cysteine side chain per polypeptide chain of the enzyme. The rate of the reaction of enzyme (5 μM polypeptide chains) with different concentrations of iodoacetamide was studied by determining the remaining activity of samples withdrawn at stated times.

	Activity remaining (%)			
	0.25 mM iodoacetamide	0.50 mM iodoacetamide	0.75 mM iodoacetamide	1.0 mM iodoacetamide
Time (min)				
0	100	100	100	100
1	95	92	88	84
2	92	84	77	71
4	84	71	60	51
8	71	51	36	26
14	55	30	17	9
20	43	18	8	3

Show that the reactions obey pseudo-first order kinetics and derive the pseudo-first order rate constants in each case and the second-order rate constant for the reaction of creatine kinase with iodoacetamide under these conditions.

4.7 Some typical K_d values for interactions of biochemical interest are avidin–biotin 10^{-15} M, antigen–antibody 10^{-10} to 10^{-9} M, and enzyme–substrate 10^{-6} to 10^{-4} M. Evaluate the ΔG^0_{310} values corresponding to these dissociation constants.

4.8 What would be the effects of competitive, non-competitive and uncompetitive inhibitors on the Eadie–Hofstee and Hanes–Woolf enzyme kinetic plots?

4.9 Chymotrypsin catalyses the hydrolysis of the ester substrate *N*-acetyl-L-tyrosine ethylester (ATEE). The reaction is inhibited by indole. The following data were obtained in the absence and presence of 1.3 mM indole. Determine the type of inhibition observed and the dissociation constant for the interaction of enzyme with the inhibitor.

[ATEE] (mM)	Velocity (μmol min^{-1} mg^{-1})	
	Without indole	With indole
2.0	9.8	3.9
4.0	14.4	5.8
6.0	17.1	6.8
10.0	20.0	8.0
15.0	21.9	8.8
20.0	23.0	9.2

4.10 Dehydroquinase catalyses the conversion of 3-dehydroquinate to 3-dehydroshikimate and water. The type II enzyme from *Helicobacter pylori* is inhibited by citrate. From the following data obtained in the absence and presence of 2 mM citrate, determine the type of inhibition observed and the dissociation constant for the interaction of enzyme with the inhibitor.

[3-dehydroquinate] (μM)	Velocity (μmol min^{-1} mg^{-1})	
	Without citrate	With citrate
50	4.4	3.0
100	6.9	5.0
150	8.5	6.5
250	10.3	8.4
500	12.4	10.9
750	13.3	12.1

4.11 Benzamidine is known to bind to the active site of trypsin, leading to inhibition of the enzyme. The binding was studied under conditions when the concentration of enzyme (3.5 μM) was very small by comparison with the concentrations of benzamidine added so that essentially all the ligand can be considered free. Using an appropriate plot, analyse the following data to determine the K_d for the trypsin–benzamidine interaction.

[Benzamidine] (mM)	*Y* (fractional saturation)
0.1	0.29
0.2	0.44
0.5	0.67
0.8	0.76
1.2	0.83
1.6	0.87

4.12 The following data were obtained during a study of the papain-catalysed hydrolysis of a model 4-nitroanilide substrate. The reaction was monitored by the increase in absorbance at 405 nm as the 4-nitroaniline product was formed. (The molar absorption coefficient for 4-nitroaniline at 405 nm is 9500 M^{-1} cm^{-1}; the substrate does not absorb at this wavelength.)

[Substrate] (mM)	Velocity (ΔA_{405} min^{-1})
0.10	0.029
0.25	0.054
0.50	0.076
0.75	0.089
1.00	0.096
1.50	0.105

The assay volume was 3 mL in a cuvette of 1-cm pathlength. The amount of papain added was 0.8 μg and the molecular mass of the enzyme is 23 kDa. Using an appropriate plot, determine the K_m for the substrate and the V_{max} for the reaction in terms of ΔA_{405} min^{-1}. Calculate the specific activity and k_{cat} for the papain-catalysed reaction.

4.13 The V_{max} for the type II dehydroquinase from *Mycobacterium tuberculosis* is 0.0134 μmol min^{-1} when 1.7 μg enzyme is added to the assay mixture. What is the k_{cat} for the enzyme? (The molecular mass of each subunit of the enzyme is 18 kDa). If the K_m for 3-dehydroquinate is 25 μM, what is the k_{cat}/K_m ratio?

4.14 The binding of ^{14}C-labelled L-phenylalanine to the enzyme pyruvate kinase was studied. After equilibrium had been achieved, the radioactivity bound to 2 mL of a solution of enzyme (0.85 mg mL^{-1}) was found to be 6250 cpm. The specific radioactivity of the ^{14}C-L-phenylalanine was 0.15 Ci mol^{-1}, and the efficiency of counting was 70%. The enzyme has a molecular mass of 240 kDa and contains four identical subunits. What is the stoichiometry of binding of L-phenylalanine to pyruvate kinase?

4.15 The amino acid methionine has the molecular formula $C_5H_{11}NO_2S$. A sample of [^{35}S] methionine was stated to have a specific radioactivity of 1.9×10^{16} Bq mol^{-1}. What per cent of the *S* atoms in the sample are present as ^{35}S? The half-life of ^{35}S is 87.4 days and Avogadro's number is 6.02×10^{23} mol^{-1}.

4.16 Ornithine decarboxylase catalyses the reaction: ornithine $\rightleftharpoons$ 1,4-diaminobutane (putrescine) + CO_2. The reaction can be monitored by measuring the release of $^{14}CO_2$ from ornithine which is labelled with ^{14}C at the carbon atom of the carboxyl group. In a reaction mixture of volume 0.4 mL, the concentration of [^{14}C]-ornithine was 10.8 μM. The specific radioactivity of the [^{14}C]-ornithine was 5.8 Ci mol^{-1}. After 10 min, the reaction was stopped and the radioactivity released as $^{14}CO_2$ was found to be 14 580 cpm; the efficiency of counting was 75%. What was the rate of the reaction expressed as nmol product formed per minute and what proportion of the original substrate present had been converted to products after 10 min?

References for Chapter 4

Price, N.C. and Stevens, L. (1999) *Fundamentals of Enzymology*, 3rd edn. Oxford University Press, Oxford, 478 pp.

Price, N.C., Dwek, R.A., Ratcliffe, R.G., and Wormald, M.R. (2001) *Principles and Problems in Physical Chemistry for Biochemists*, 3rd edn. Oxford University Press, Oxford, 401 pp.

Appendix

Appendix 4.1 Binding of ligands to macromolecules: saturation curves

Consider the binding of ligand L to a protein P which has a single binding site:

$$P + L \rightleftharpoons PL$$

For which $K_d = \dfrac{[P] \times [L]}{[PL]}$, hence $[PL] = \dfrac{[P] \times [L]}{K_d}$

At a given concentration of free ligand ($[L]$), the fractional saturation of the binding sites (Y) equals the concentration of bound ligand divided by the total concentration of protein.

Hence $Y = \dfrac{[PL]}{[P] + [PL]}$

$$Y = \frac{\dfrac{[P] \times [L]}{K_d}}{[P] + \dfrac{[P] \times [L]}{K_d}}$$

Dividing each term in the numerator and denominator by $[P]$:

$$Y = \frac{\dfrac{[L]}{K_d}}{1 + \dfrac{[L]}{K_d}}$$

Multiplying each term in the numerator and denominator by K_d

$$Y = \frac{[L]}{K_d + [L]} \qquad 4.22$$

When there are n binding sites for ligand on the protein, the fractional saturation equation is analogous to eqn. 4.22. It is expressed in the following terms:

$$r = \frac{n \times [L]}{K_d + [L]} \qquad 4.23$$

where r is the average number of molecules of ligand bound per molecule of protein, n the number of binding sites, $[L]$ is the concentration of free ligand, and K_d is the dissociation constant.

It is, however, considerably more complex to derive this equation since it is necessary to take into account the statistical nature of the relationship between the successive dissociation constants for binding of ligand. (This is discussed in Chapter 6 of the book by Price and Stevens (1999)).

In the case of enzyme kinetics we can consider the fractional saturation of the active sites of the enzyme by the substrate S. One way of calculating the fractional saturation is to make the assumption that in the mechanism $E + S \rightleftharpoons ES \rightarrow E + P$ the breakdown of ES to yield E + P does not perturb the $E + S \rightleftharpoons ES$ equilibrium significantly. In this case, the fractional saturation can be written as:

$$Y = \frac{[S]}{K_d + [S]}$$

In this case the K_d is denoted by the K_m (Michaelis constant), so:

$$Y = \frac{[S]}{K_m + [S]}$$

Complete saturation of the active sites corresponds to the limiting rate (V_{max}), so the actual rate (v) at a substrate concentration ($[S]$) is given by $\frac{v}{V_{max}}$, which corresponds to the fractional saturation, i.e. Y.

$$\text{Hence } \frac{v}{V_{max}} = \frac{[S]}{K_m + [S]}, \text{ so}$$

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \qquad 4.26$$

We can also derive an equation of this form by making the steady state assumption, namely that the concentration of the intermediate (ES) remains constant, i.e. the rate at which it is formed equals the rate at which it is broken down. In this case, K_m represents a function of various rate constants of the individual steps in the reaction. Only in the limiting case (i.e. that the rate of ES breakdown to give E + P equals zero) does K_m become equal to the dissociation constant of ES to give E + S. (For further details see Chapter 4 of Price and Stevens (1999).)