The Effect of pH on the Affinities of Enzymes for Substrates and Inhibitors

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There are five types of equilibria, representing reversible combinations, which are especially important in biochemistry. They are as follows (see Table 1): (a) acid-base equilibria: combination of acid anions with hydrogen ions; (b) oxidation-reduction equilibria: combination of oxidants with hydrogen; (c) phosphorylation equilibria: combination of organic molecules with inorganic phosphate; (d) Michaelis complex formation: combination of enzymes with their substrates; (e) inhibition equilibria: combination of enzymes with reversible inhibitors.

All these equilibria are formally similar; they obey equations which are identical in form, and are amenable to the same treatment. It has been shown (Dixon, 1949) in the case of the hydrogenation and phosphorylation equilibria that when the systems are plotted on a logarithmic scale with respect to the second reactant shown in column 2 of Table 1 (H_2 or P respectively), a number of simple and useful rules can be formulated which greatly facilitate the interpretation of the experimental data. It is the purpose of the present paper to show that if the systems of the last two types are similarly represented, rules of identical form can be applied to them. It appears probable that these rules may throw a good deal of light on the mechanism of enzyme-substrate and enzyme-inhibitor combination and on the nature of the chemical groups involved, and that a profitable new field for enzyme work may be opened up.

THEORY

Scale definitions

For the treatment of the five types of equilibria (Table 1), five different but analogous scales are already in use, represented by the symbols pH , rH , rP , pS and pI respectively. In each case the scale value is defined as $-\log_{10} c$, where c is the concentration (or more strictly the activity) of the 'scale-defining substance', i.e. the second reactant shown in column 2. For hydrogen the activity is expressed in atmospheres, for the other cases, in molarities. The scale definitions are shown in column 3 of the table.

No new symbols are required and these symbols are used with their accepted meanings. It will be

noticed as we proceed that when the various sets of data are brought together in such a unified scheme the symbols do not form as consistent a system as one could wish. The reason is that they have been introduced at different times by a number of workers, all concerned with different systems.

There is a slight theoretical difference between the last two types and the others. For each of the first three types the scale-defining substance is the same for all the systems in that group. All acid anions are in equilibrium with hydrogen ions, all the hydrogenated systems with hydrogen and all the phosphorylated systems with phosphate. All enzymes, however, are not in equilibrium with the same substrate; on the contrary, we are concerned with a different substrate for each enzyme. But this point is of no importance in practice, for we are almost invariably concerned with only one enzyme at a time in this connexion, and have not to consider the distribution of a given substrate between a number of enzymes.

Effect of relative concentrations of components

Putting the equilibrium equations into their logarithmic forms we obtain the following five equations

$$\left. \begin{aligned} pH &= pK - \log \frac{[AcH]}{[Ac^-]}, \\ rH &= rH_0 - \log \frac{[AH_2]}{[A]}, \\ rP &= rP_0 - \log \frac{[XP]}{[X]}, \\ pS &= pK_{s0} - \log \frac{[ES]}{[E]}, \\ pI &= pK_{i0} - \log \frac{[EI]}{[E]}. \end{aligned} \right\} \quad (1)$$

If therefore we plot the first of these quantities in each case against the composition of the systems, i.e. the degree of reduction, phosphorylation, etc., we obtain in every case the same curve of the familiar form shown in Fig. 1. This curve represents (a) the degree of association of the acid in terms of the pH , (b) the degree of reduction (or hydrogenation) of the

Table 1. *Formulations and symbols for various equilibria*

Column 1	2	3	4	5
Type	Equilibrium	Scale definitions	Mid-point without ionization	Mid-point with ionization
(a) Acid-base	$Ac^- + H^+ \rightleftharpoons AcH$	$pH = -\log [H^+]$	pK	—
(b) Oxidation-reduction	$A + H_2 \rightleftharpoons AH_2$	$rH = -\log [H_2]$	rH_0	$r'H$
(c) Phosphorylation	$X + P \rightleftharpoons XP$	$rP = -\log [P]$	rP_0	$r'P$
(d) Enzyme-substrate	$E + S \rightleftharpoons ES$	$pS = -\log [S]$	pK_{s0}	pK_s
(e) Enzyme-inhibitor	$E + I \rightleftharpoons EI$	$pI = -\log [I]$	pK_{i0}	pK_i

oxidation-reduction system in terms of the rH , (c) the degree of phosphorylation of an organic system in terms of the rP , (d) the degree of 'substitution' of an enzyme (if we may coin a word) in terms of the pS , and (e) the degree of inhibition of an enzyme in terms of the pI .

Fig. 1. Effect of alteration of composition of the five types of system (Table 1). The arrow represents the effect of ionization.

When there are no complications due to the effects of ionization of the components, which will be dealt with below, the mid-points of the curves are given by the middle terms of equations (1), as shown in column 4 of the table. The symbols have their usual meanings, and pK_{s0} and pK_{i0} denote minus the logarithm of the Michaelis constant and inhibitor constant respectively, the subscripts '0' showing that the quantities refer to the non-ionized systems. The term 'Michaelis constant' is used in this paper

in its original sense as defined by Michaelis & Menten (1913), i.e. as the dissociation constant of the enzyme-substrate complex, or in other words the reciprocal of the enzyme-substrate affinity. To avoid any confusion the earlier symbol K_s for this equilibrium constant will be used here, rather than the commonly used K_m , as in recent years the latter has come to be used to denote the substrate concentration giving half the maximum velocity, a less fundamental but more easily measured quantity which may be partly kinetic in nature. K_s and K_m are equal, except in those cases which require the use of the Briggs-Haldane (1925) theory, where the kinetic element in K_m causes it to diverge from the true equilibrium constant K_s .

Free energy and heat of formation of ES and EI complexes

The same rules as for rH and rP apply (see Dixon, 1949, pp. 70, 72, 93-4; rules 3 and 8). Thus the standard free energy of formation of the Michaelis complex is given by $-\Delta G^\circ = 2.3RT \cdot pK_{s0}$ (or, if there is ionization, by $2.3RT \cdot pK_s$ (see next section) the value then including the free energy of the ionization reactions).

The heat of combination of enzyme with substrate is given by $\Delta H = 2.3RT^2 \frac{d(pK_s)}{dT}$.

Similar rules, with substitution of K_i for K_s , apply to the formation of inhibitor-enzyme complexes.

Effect of ionization of the components

In the case of rH and rP it has been shown that ionization of one or more of the components of the system causes a displacement of the curve to an extent depending on the pH , so that the mid-point is shifted along the scale from the position (rH_0 or rP_0) which it had in the absence of ionization effects, to new positions denoted by $r'H$ and $r'P$ respectively, as shown by the arrow in Fig. 1. These effects are revealed when we plot the values of the half-hydrogenated or the half-phosphorylated system (i.e. $r'H$ or $r'P$) against the pH . In what follows we are no longer concerned with the first line of the table, since it is meaningless to plot pH against pH .

The deviations caused by ionization are due to the fact that when there is ionization the amount of the unionized form of a given component (to which equations (1) refer) is no longer equal to the total amount of the component taken, because the total concentration is the sum of the concentrations of the unionized form and all the ionized forms. It is therefore necessary to obtain an expression for the concentration of the unionized form of the substance in terms of its total concentration, its ionic dissociation constants and the pH, and to substitute this in equations (1).

Taking an rH system as an example, the molecule of the reduced component AH_2 (which might represent some such substance as quinol) might ionize as a dibasic acid with dissociation constants K_1 and K_2 . Its total concentration would then be given by $Re_t = [AH_2] + [AH^-] + [A^{2-}]$ and therefore $Re_t = [AH_2] \cdot f(pH)$, where $f(pH)$ is a function of the pH and the dissociation constants of AH_2 . Substituting in the second of equations (1) we get

$$rH = rH_0 - \log \frac{Re_t}{Ox_t} + \log f(pH).$$

For the half-reduced system $Re_t = Ox_t$, so that $r'H = rH_0 + \log f(pH)$ and the last term represents the amount of the deviation of the mid-point from rH_0 due to the ionization. Following Michaelis (1930) we shall use the subscript t to denote the total concentration of all ionization states of a given substance.

In the case of a dibasic acid like quinol, $\log f(pH)$ is equal to $\log \left(1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2} \right)$ (see Dixon, 1949, p. 74).

The treatment of the enzyme systems is exactly analogous. The substrate, the enzyme and the enzyme-substrate complex may all ionize. In order to illustrate the treatment let us assume, for the sake of simplicity, that each has two acidic groups like quinol. Then if E_t denotes the total concentration of free enzyme (not the total enzyme concentration) and S_t the total free substrate concentration,

$$\left. \begin{aligned} E_t &= [E] + [E^-] + [E^{2-}] = [E] \left(1 + \frac{K_{e1}}{[H^+]} + \frac{K_{e1} K_{e2}}{[H^+]^2} \right) = [E] \cdot f_e(pH), \\ S_t &= [S] + [S^-] + [S^{2-}] = [S] \left(1 + \frac{K_{s1}}{[H^+]} + \frac{K_{s1} K_{s2}}{[H^+]^2} \right) = [S] \cdot f_s(pH), \\ ES_t &= [ES] + [ES^-] + [ES^{2-}] = [ES] \left(1 + \frac{K_{es1}}{[H^+]} + \frac{K_{es1} K_{es2}}{[H^+]^2} \right) = [ES] \cdot f_{es}(pH). \end{aligned} \right\} \quad (2)$$

K_{e1} , K_{e2} (the acidic dissociation constants of the substrate) should not be confused with K_s .

The ordinary Michaelis constant K_s is the equilibrium constant of the enzyme-substrate complex formation, expressed in terms of the total

concentrations of the components, whereas the constant K_{s0} which appears in the fourth of equations (1) is the corresponding constant for the unionized forms, therefore

$$K_s = \frac{E_t \cdot S_t}{ES_t} = \frac{[E][S] f_e(pH) \cdot f_s(pH)}{[ES] \cdot f_{es}(pH)} = K_{s0} \frac{f_e(pH) \cdot f_s(pH)}{f_{es}(pH)},$$

or in logarithmic form

$$pK_s = pK_{s0} + \log f_{es}(pH) - \log f_e(pH) - \log f_s(pH). \quad (3)$$

The functions $f(pH)$ are identical with those appearing in the corresponding rH and rP equations, having the form $1, 1 + \frac{K}{[H^+]}, 1 + \frac{K_1}{[H^+]} + \frac{K_1 K_2}{[H^+]^2}$, etc., according to whether the substance has 0, 1, 2, etc., ionizing groups. With the appropriate substitution of symbols, equation (3) is identical with that previously given for the other type of system (cf. Dixon, 1949, p. 95).

The treatment does not imply any assumption about the actual mechanism of combination, or which particular ionic form of the substrate it is which combines with the enzyme. It is simply assumed that there is a relation between the concentrations of the unionized components such that $\frac{[E][S]}{[ES]} = \text{constant}$. But since all the ions are in equilibrium with the unionized forms, this implies that there are also similar equilibrium relationships between the corresponding ionic forms. It is easy to show by calculation that the same result is obtained whatever mechanism of combination is assumed.

When there is ionization, the mid-points of all the curves are given by the quantities given in column 5 of Table 1. pK_s , being the point at which the enzyme is 'half-substrated' (or at which $E_t = ES_t$), corresponds to $r'H$ and $r'P$, the points at which the other systems are half-hydrogenated and half-phosphorylated respectively, and, on plotting pK_s against pH, graphs are obtained which obey the same rules as the $r'H$ and $r'P$ charts previously

given, because the functions $f(pH)$ are the same for all systems which ionize similarly (Dixon, 1949, pp. 73 and 98).

These graphs consist of sections of straight lines joined together, each straight part covering a range

of pH in which one of the terms in the functions $f(\text{pH})$ is large compared with the others. Each section will have a zero, 1-unit or 2-unit slope in agreement with the power of $[\text{H}^+]$ occurring in the term which is dominant at that pH, and the slope will be positive or negative according to the sign preceding the function in which the term occurs in equation (3). Intermediate values of slope are of course met with in the short curved part at the junction of two sections, where two of the terms are important at the same time. If more than one component of the system ionizes, so that the equation contains two or three pH functions, as in equation (3), the slope will be the algebraic sum of the contributions of all the functions. The term '+1-unit slope' means that the pK_s increases by 1 unit for every increase of 1 unit in the pH.

A very simple example may make this clearer. Let us suppose that the enzyme and the $E\text{-}S$ complex do not ionize, but that the substrate has one ionizing group with a pK of 7. On the acid side, well below the pK value, the substrate will be present almost entirely as the unionized form. On the alkaline side, well above the pK , it will be present almost entirely as the anion S^- . Near the pK the substrate will be present as a mixture of the two forms S and S^- in varying proportions.

The functions $f_s(\text{pH})$ and $f_{ss}(\text{pH})$ will both be equal to 1 and their logarithms will be zero; $f_s(\text{pH})$ will be $1 + K/[\text{H}^+]$. Equation (3) therefore takes the form

$$\text{pK}_s = \text{pK}_{s0} - \log \left(1 + \frac{10^{-7}}{[\text{H}^+]} \right).$$

Below pH 5, $10^{-7}/[\text{H}^+]$ is negligible compared with 1; pK_s is therefore equal to pK_{s0} and independent of the pH in the region where the change on combination is from $E + S$ to ES . Above pH 9, $10^{-7}/[\text{H}^+]$ is large compared with 1 and the equation will become $\text{pK}_s = \text{pK}_{s0} - (\text{pH} - 7)$. This represents a straight line with a -1 -unit slope intersecting the pK_{s0} line at pH 7, the pK value. In this -1 -slope region the change on combination is from $E + S^-$ to ES . In the neighbourhood of the pK neither of the terms within the bracket can be neglected and there is a curved region of the graph. At pH 7, $10^{-7}/[\text{H}^+]$ equals 1, and the curve is therefore $\log 2$ or 0.3 unit below the point of intersection. The graph is of the form shown in Fig. 2A.

The rules which permit the easy interpretation of these pH graphs are two in number, the 'rules 9 and 10' of Dixon. The first states that the slope in any pH region is numerically equal to the change of charge on dehydrogenation, dephosphorylation or 'desubstration'; that is to say it is equal to the number of charges on the left-hand side of the equations in column 2 of Table 1 minus the number of charges on the right-hand side, due regard being

paid to algebraical sign. For example, in the case of rH, quinol is present in alkaline solutions as the anion Q^{2-} , and the oxidation may be represented as $\text{Q}^{2-} \rightarrow \text{Q}$, a change of $+2$ (from two negative charges to zero), and the system gives a $+2$ -unit slope on the rH chart in this pH range. At pH 8 the dephosphorylation of ATP takes place as follows: $\text{ATP}^{4-} \rightarrow \text{ADP}^{3-} + \text{P}^{2-}$, a change of -1 , giving a -1 -unit slope on the rP chart.

Fig. 2. Diagram to illustrate various effects of pH (see text).

Correspondingly in the case of enzyme systems, if there is no alteration of total charge when the substrate combines with the enzyme, pK_s will not change with pH and the graph will be a horizontal straight line. This would be expected for instance with a non-ionizing substrate, e.g. a carbohydrate, combining with a non-ionized group in the enzyme. It would also be expected where the combination is electrostatic in nature, e.g.

On the other hand, if an acidic substrate were to combine through its acidic group with the enzyme to give a non-ionized compound

there will be a charge change of -1 on 'desubstration' and a -1 -unit slope will be expected in the pH range in which the substrate is present as the anion. On the acid side of the pK value of the substrate, however, the substrate will be unionized and the graph will become horizontal as shown in Fig. 2A. pK relates to the acid-base ionization of the components and should not be confused with

pK_s or pK_i . Curves of this form have recently been obtained with inhibitors in two cases. Massey (1953) finds that maleate acting on fumarase gives such a curve, with the bend corresponding to the pK of maleate. Chance (1952) gives similar curves for the inhibitors hydrazoic acid and hydrocyanic acid acting on catalase in which the bends agree approximately though not exactly with the respective pK values; in this case the enzyme-inhibitor combination was measured directly by a spectrophotometric method.

This brings us to the second rule, 'rule 10', which states that every change of direction of the graph corresponds to the ionization of a group in one of the components; when the upper side of the bend is the concave side, it indicates a pK (acidic or basic) of the enzyme-substrate complex, while when the concave side faces downwards it indicates a pK of either the free substrate or the free enzyme. This rule can be shown to follow from the fact that in equation (3) the functions $f(pH)$ appear with a positive sign when they refer to substances on the right-hand side of the equilibrium equations in column 2 of Table 1, and with a negative sign when they refer to substances on the left-hand side. Each ionizing group produces a change of one unit in the slope when the pH passes through its pK value and the value of the pK is given by the point of intersection of the straight parts produced, as shown in Fig. 2*A*. In this example, as already mentioned, the ionizing group is present in the free substrate, and it will be noted that the concave side of the bend faces downwards in agreement with the rule.

The curvature at the junction is such that the graph passes through a point 0.3 pK_s unit above or below the point of intersection of the straight parts (Dixon, 1949, p. 76) as shown in Fig. 7. In some cases, however, for reasons which are as yet unexplained, the experimental curves seem to give sharper bends than the theoretical (cf. Fig. 4 and Massey, 1953).

It may seem at first sight that the interpretation of such graphs is subject to ambiguities, since there is nothing to show whether a downward bend represents a pK of the free substrate or of the free enzyme. There is little trouble in practice, however, because in the great majority of cases the pK values of the substrate are known, and downward bends occurring at any other pH values must represent ionizations of the free enzyme.

What is meant by ionization of the enzyme may not be immediately obvious, since, as a protein, it will naturally contain many ionizing groups. It has been pointed out already in the case of the rH systems that groups which ionize equally strongly in the reduced and oxidized forms, e.g. sulphonate groups in dyes, have no effect on the curves. They are not detected and can be disregarded. This is

evident from the rule just stated, since groups with the same pK values in the oxidized and reduced forms will produce equal and opposite bends in the curve which will cancel one another out. The same is true in the case of enzymes; a group in the enzyme protein which is equally strongly ionized in the free enzyme and the enzyme-substrate complex will not be shown by the curve. It is only groups whose degree of ionization alters when the enzyme combines with substrate, i.e. groups which form part of the active centre or are sufficiently closely associated with it to be affected when the substrate combines, which are revealed by the curves. This fortunate circumstance enables us to obtain, from the plots of pK_s against pH, information about the active centres, uncomplicated by the ordinary unspecific ionizations which all proteins undergo.

Thus it is essentially the substrate-binding groups in the enzyme which are revealed by the method. It does not follow, however, that all such groups will be shown up. Any substrate-binding group which does not ionize at any accessible pH, either in the free enzyme or the complex, will produce no discontinuities in the curve from which suggestions as to its nature can be obtained.

Various effects may be produced by ionizing groups. If the substrate does not actually combine with the group in question, but merely affects its ionization so that its pK is not the same in the enzyme-substrate complex as it is in the free enzyme, a wave will appear in the curve, as shown in Fig. 2, curve *B*, corresponding to an increase of pK by the substrate and curve *C* to a decrease. It is easily shown that the overall difference in level is then numerically equal to the change in pK . The substrate may combine with an ionized group in the enzyme, forming a non-ionized link, in which case there will be a single downward bend at the pK of the group, giving a curve similar to *A*. On the other hand, a group which is not ionized in the normal substrate may ionize when the substrate becomes activated by combination with the enzyme, giving an upward bend.

The curves may be expected to yield valuable information of three kinds. (a) Information about the nature of the enzyme-substrate link, deduced mainly from the slope of the curve: for instance it has been suggested that alkaline phosphatase combines with its substrate by an electrostatic attraction between the negatively charged ionized phosphate group of the substrate and the positively charged Mg atom, somewhat as follows:

Since there is no alteration of the total charge, a zero slope would be expected. On the other hand, if one of the ionized OH groups of the substrate combines with the enzyme with the formation of a

non-ionized link, $R.P^{2-} + E \rightarrow R.P^-.E$, a -1-unit slope would be expected, and this is what is found in the pH range in which the enzyme is most active.

(b) Information about the nature of the substrate-binding groups of the enzyme, deduced from their pK values as determined from the position of the discontinuities of the curve: due caution is of course necessary here; the fact that a substrate-binding group in the enzyme has a pK which is identical with the known pK of some particular group which occurs in proteins is not of itself proof of its nature, because other groups may possibly be found which have the same value of pK . Nevertheless, valuable suggestions may be obtained in this way. Phosphatase is again a case in point, as shown below.

(c) Information about the nature of the activation process, deduced from the ionizations of the enzyme-substrate complex: it is not impossible that in certain cases when the substrate becomes activated by the enzyme, certain groups in its molecule may become ionized which normally are non-ionizing. For instance, according to Quastel's (1926, p. 177) theory of the activation of succinic acid by its dehydrogenase, the methylene groups of the activated molecule ionize thus

and this would be shown by discontinuities in the pK_s curve referable to the enzyme-substrate complex. It is not known whether this is the case.

In order to avoid any misunderstanding it should be pointed out again that the data do not show which of the various forms of the substrate it is which actually combines with the enzyme. The treatment is based on thermodynamic equilibria and does not give the actual mechanism of combination. It gives the overall change of charge when the combination takes place, and the result is precisely the same if the enzyme combines in the first place with, say, the substrate ion as if the unionized substrate first combines and then ionizes. The slope is determined by the forms of the components which are actually present at any pH.

It will be clear that what has been said about enzyme-substrate combination in relation to the pK_s curves applies equally to the reversible combination of enzymes with inhibitors in relation to pK_i curves. The method should yield the same kind of information about inhibitors as about substrates. Indeed it is possible that the study of inhibitors in this way may yield more information about enzymes than the use of substrates, since with any given enzyme the number of inhibitors available for study is usually much larger than the number of substrates. Moreover, it should be possible to obtain information about the groups in the enzyme which combine with particular parts of

the substrate molecule by the use of inhibitors containing these parts. This appears to offer a wide field for research.

A comparison of pK_s and pK_i curves for the same enzyme should further assist interpretation, since (unless the presence of the substrate or inhibitor changes the state of the enzyme in some way) the free enzyme will have the same charge in all cases and its pK values should be the same in all the curves.

Measurement of K_s and K_i

The preceding sections have been concerned with the theory of the effect of pH on the affinities of enzymes for substrates and inhibitors; they have therefore been concerned only with the equilibria between the components. The practical measurement of these affinities is an entirely separate question, and cannot be dealt with fully within the scope of this paper. Nevertheless, a brief summary of the position may not be out of place here.

Direct measurements of enzyme affinities are possible in certain cases, e.g. where there is a change in absorption spectrum when combination with the enzyme takes place. With enzymes which can be obtained in the pure state in reasonably large amounts it should also be possible to measure the combination by direct chemical estimations in certain cases.

The most widely used methods, however, depend upon measurements of the velocity of the enzyme-catalysed reaction, and the kinetic element in these measurements must not be lost sight of. This kinetic element may in some cases give rise to difficulties in the determination of K_s , though not of K_i . Difficulties in the measurement of K_s do not affect the validity of the theory in any way, though they may sometimes affect its applicability.

The method most generally used for studying affinities for substrates is known as the Lineweaver & Burk graph (1934), which gives K_m , the substrate concentration at which the velocity is half the maximal velocity obtained with high substrate concentration. Whether this is equal to K_s , as would follow from the Michaelis treatment, or whether it diverges from it because of kinetic effects, depends on the relative magnitude of the velocity constants of the system. Writing the reaction as

it will be clear that if k_3 is small compared with k_1 and k_2 , ES will at all times be in equilibrium with E and S . This was the basic assumption of the Michaelis theory, and under these conditions K_m is equal to K_s (i.e. k_2/k_1). In such cases the theory given in this paper, which relates to K_s , can be applied directly to the values of K_m determined by

the Lineweaver method. There is no difficulty in such cases.

In those cases where k_3 is not relatively small, Briggs & Haldane (1925) showed that the observed K_m is equal to $(k_2 + k_3)/k_1$ and therefore differs from K_1 . It must be emphasized, therefore, that the direct application of the rules given above to the observed K_m values may lead to errors in those cases in which it is necessary to invoke the Briggs-Haldane theory. It may well be, however, that the errors will be confined to a small region in the neighbourhood of the optimum pH where k_3 is large.

Where it is possible to vary k_3 experimentally, e.g. by varying the concentration of a second reactant, K_s may obviously be obtained by extrapolating K_m to $V = 0$, V being the maximal velocity obtained in high substrate concentration, which is proportional to k_3 . Slater & Bonner (1952) have made use of this principle to determine K_s for succinic dehydrogenase.

Even when the absolute value of K_s cannot be determined it is still possible to obtain some information from the rules by considering together the variation with pH of pK_m and pV (i.e. $-\log V$). It is hoped to deal with the application of the theory to Briggs-Haldane cases more fully in a later paper, but three points may be mentioned here. (1) Rule 10 is still applicable, since the discontinuities in the pK_s graph will also appear in the pK_m graph. (2) In pH regions where the pV curve is horizontal, the participation of k_3 makes no difference to the application of rules 9 and 10, because even though K_m is not equal to K_s , calculation shows that the pK_m graph is parallel to the pK_s graph and is merely displaced from it in the vertical direction. Thus within such regions the slopes are the same and the rules can be applied to the Briggs-Haldane case no less than to the Michaelis case. (3) The occurrence in the pK_m graph of straight portions having whole-number slopes (e.g. zero or 1-unit slopes), in pH regions where the pV graph has non-whole-number slopes or is curved, indicates that k_3 is negligible and the theory can be directly applied to the pK_m graphs in such regions. This is a useful criterion of applicability.

This extension of the theory is still being developed. The grounds for these statements will be given later: they are included here in order to give some idea of the range of applicability of the rules.

No such difficulties arise in the determination of K_i , since the Michaelis equation on which it is based is valid both for the Briggs-Haldane and the Michaelis cases. The reversible inhibitor comes quickly into equilibrium with the enzyme, since the EI complex is not used up and there is nothing corresponding to k_3 for the inhibitor. K_i , the actual dissociation constant of the enzyme-inhibitor

complex, is conveniently determined by the graphical method of Dixon (1953).

EXAMPLES

A search of the literature shows that experimental work on the variation of Michaelis constants with pH is extremely scanty; in fact the field seems to be almost untouched. The Michaelis constants of a great many enzymes have been determined, but nearly always at one pH value only. In a few cases measurements have been done at two or more pH values, but they are too few, or cover too restricted a pH range, to allow curves to be drawn. The writer has only been able to find seven cases in which the effect of pH on K_m has been studied, namely urease, phosphatase, arginase, saccharase, fumarase, cholinesterase and xanthine oxidase.

Fig. 3. Effect of pH on pK_m of urease. Plotted from data of Howell & Sumner (1934).

Urease. Howell & Sumner (1934) give a series of curves showing the relation between urea concentration and velocity at different pH values in 0.0125M-phosphate. The Michaelis constants read from these curves have been plotted as pK_m in Fig. 3, and it will be seen that they fall on a straight line with a +1-unit slope. This would mean either that the enzyme loses one positive (or gains one negative) charge when it combines with urea, or that when the urea is activated by combination with the enzyme it acquires a negative charge. In interpreting these results, however, caution is necessary, because they are only obtained in the presence of

phosphate. In the absence of phosphate the affinities are very much higher and cannot be determined from Howell & Sumner's curves; under these conditions therefore the effect of pH is unknown.

Phosphatase. The lower curve of Fig. 4 is plotted from the data of Jacobsen (1933) on the alkaline phosphatase of kidney, with glycerolphosphate as substrate. A -1-unit slope is obtained, as already mentioned. Recently Morton (1952) has studied highly purified intestinal phosphatase with phenylphosphate as substrate, obtaining the upper curve in Fig. 4. This shows a -1-unit slope on the alkaline

Fig. 4. Effect of pH on pK_m of alkaline phosphatase. Lower curve for kidney phosphatase with glycerolphosphate, plotted from data of Jacobsen (1933); upper curve for highly purified intestinal phosphatase with phenylphosphate, from Morton (1952).

side, but has a discontinuity at about pH 9.2 which is not seen in the other curve. This cannot represent the pK of the substrate, which lies at 5.73 (Delory & King, 1943), and must therefore be due to an ionizing group in the enzyme. The pK value would correspond with a phenolic tyrosine group, an ϵ -amino group, or an SH group; there is independent evidence, however, that SH groups are not involved. It is not certain whether the difference between the two curves is due to the use of different enzymes or of different substrates. The latter seems more probable, for there are indications of a similar discontinuity in the results of Folley & Kay (1935) who used milk phosphatase with phenylphosphate. If so, it suggests that the group in question is concerned in the combination of the enzyme with phenylphosphate but not with glycerolphosphate.

Arginase. The curve shown in Fig. 5 has been plotted from the results of Mohamad & Greenberg (1945) and includes results obtained with ox and

Fig. 5. Effect of pH on pK_m of arginase. Plotted from data of Mohamad & Greenberg (1945).

Fig. 6. Effect of pH on pK_m of saccharase. Plotted from data of Josephson (1924).

rabbit arginase. These authors plot values of K_m and draw a smooth curve, but actually all the points between pH's 7 and 9 lie on a horizontal straight line and not on the curve. Their interpretation of the

results, which is no doubt correct, is that in the central range of pH a negatively charged group in the enzyme combines with the positively charged amino group of the arginine, which should give a zero slope; the slope on the alkaline side is then due to the amino group of the substrate (pK about 9) and on the acid side to the group in the enzyme (to which they assign a pK of 7).

Saccharase. In this case the substrate does not ionize. Josephson (1924) states that the affinity of saccharase for sucrose is constant between pH's 4 and 8, but falls off in more acid solution. The experimental points are not sufficiently numerous to draw an accurate curve, but as shown in Fig. 6 they

Fig. 7. Effect of pH on pK_m of cholinesterase. Plotted from data of Hase (1952).

indicate that one of the substrate-binding groups in the enzyme has a pK of about 3. It has been suggested that this group is an amino group, but the pK value makes this unlikely and would correspond better with a carboxyl group. Of course this may not be the only substrate-binding group and the sugar may combine at the same time with other groups which do not ionize, at any rate within this pH range.

Fumarase. The effect of pH on the affinity of pure crystalline fumarase for substrates and inhibitors has recently been studied in detail by Massey. Some complications are introduced by the fact that several of the substances act also as activating anions, but the results, which are being reported separately (Massey, 1953), are of much interest and should throw light on the nature of the active centre of the enzyme and its mode of action.

Cholinesterase. The effect of pH on the action of horse serum on acetylcholine has been studied by Hase (1952), and his results on the K_m have been plotted in Fig. 7. He draws attention to the fact that the tangent of the graph in the more acid region is 1, and ascribes the bend, no doubt correctly, to an ionization of the enzyme with a pK of 7.7. As the substrate is positively charged throughout this pH range, the data suggest a combination $E^- + S^+ \rightarrow ES$ (zero slope) on the alkaline side and $E + S^+ \rightarrow ES$ (+1-unit slope) on the acid side. It may be mentioned, in order to avoid confusion, that this author uses the symbol ϕ_s for our K_m and that he uses the symbol ' K_m ' with a different meaning.

Fig. 8. Effect of pH on pK_m of xanthine oxidase acting on 2-amino-4-hydroxypteridine. Plotted from data of Lowry, Bessey & Crawford (1949). The dotted line shows a possible interpretation.

Xanthine oxidase. Lowry, Bessey & Crawford (1949) have studied the oxidation of 2-amino-4-hydroxypteridine by xanthine oxidase and their data are plotted in Fig. 8. If we assume pK's for the free enzyme (or substrate) of 5.3 and 7.9, and one for the complex of 4.4, the theory would give the curve drawn, which agrees quite well with the experimental points. In the absence of further data, however, this interpretation should not be taken as more than a suggestion.

SUMMARY

It is shown that if the affinities of enzymes for substrates and inhibitors are expressed on a logarithmic scale (pK_s or pK_i) the data can be interpreted by

simple rules. These rules, which are identical in form with those already worked out for oxidoreductions and phosphorylations (Dixon, 1949), enable deductions about the nature of the enzyme-substrate or enzyme-inhibitor combination and the

ionization constants of the groups involved to be drawn from the effect of pH upon the affinities.

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REFERENCES

- Briggs, G. E. & Haldane, J. B. S. (1925). *Biochem. J.* **19**, 338.
 Chance, B. (1952). *J. biol. Chem.* **194**, 483.
 Delory, G. E. & King, E. J. (1943). *Biochem. J.* **37**, 547.
 Dixon, M. (1949). *Multi-enzyme Systems*. Cambridge University Press.
 Dixon, M. (1953). *Biochem. J.* **53**, 329.
 Folley, S. J. & Kay, H. D. (1935). *Biochem. J.* **29**, 1837.
 Hase, E. (1952). *J. Biochem., Tokyo*, **39**, 259.
 Howell, S. F. & Sumner, J. B. (1934). *J. biol. Chem.* **104**, 619.
 Jacobsen, E. (1933). *Biochem. Z.* **267**, 89.
 Josephson, K. (1924). *Hoppe-Seyl. Z.* **134**, 50.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Lowry, O. H., Bessey, O. A. & Crawford, E. J. (1949). *J. biol. Chem.* **180**, 399.
 Massey, V. (1953). *Biochem. J.* **55**, 172.
 Michaelis, L. (1930). *Oxidation-Reduction Potentials*. Philadelphia and London: J. B. Lippincott Co.
 Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
 Mohamad, S. M. & Greenberg, D. M. (1945). *Arch. Biochem.* **8**, 365.
 Morton, R. K. (1952). Ph.D. Thesis, Cambridge University.
 Quastel, J. H. (1926). *Biochem. J.* **20**, 166.
 Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.

The Determination of Enzyme Inhibitor Constants

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The Michaelis constant, K_m , that is to say the equilibrium constant of the reversible combination of an enzyme with its substrate, is most conveniently determined by Lineweaver & Burk's (1934) method of plotting. The great advantage of this method is that by plotting $1/v$ against $1/s$ it makes it possible to represent the Michaelis equation

$$v = \frac{Vs}{K_m + s} \quad (1)$$

by a straight line. Here v is the reaction velocity with the substrate concentration s , and V is the maximum velocity obtained at high substrate concentrations. Lineweaver & Burk calculate K_m from the slope of the line and its intercept on the vertical axis, but there is a simpler method which they do not mention, namely to produce the line to the left of the vertical axis, when it will cut the baseline at a point giving $-1/K_m$, as shown in Fig. 1. This is easily shown by putting $1/v = 0$ in the reciprocal form of equation (1), namely

$$\frac{1}{v} = \frac{K_m}{V} \frac{1}{s} + \frac{1}{V},$$

which then gives $1/s = -1/K_m$.

The inhibitor constant, K_i , that is to say the equilibrium constant of the reversible combination of the enzyme with a competitive inhibitor, has hitherto usually been obtained by calculation from the Michaelis equation for a competitive system

$$v = \frac{Vs}{K_m \left(1 + \frac{i}{K_i}\right) + s}, \quad (2)$$

where i is the inhibitor concentration. For this calculation the effect on the velocity of varying independently both s and i must be determined.

There is, however, a simple graphical method of determining K_i , which, as far as the writer is aware, has not been described previously. If $1/v$ is plotted against i , keeping s constant, a straight line will be obtained, and if this is done at two different substrate concentrations s_1 and s_2 the lines will cut one another at a point on the left of the vertical axis, as shown in Fig. 2. This point lies at $-K_i$, which can therefore be read off directly.

The proof is as follows. Each line represents the reciprocal form of equation (2), namely

$$\frac{1}{v} = \frac{K_m}{Vs} + \frac{1}{V} + \frac{K_m}{Vs} \frac{i}{K_i}. \quad (3)$$