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Estimation of the Dissociation Constants of Enzyme–Substrate Complexes from Steady-State Measurements

INTERPRETATION OF pH-INDEPENDENCE OF K_m

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If the Michaelis constant of an enzyme-catalysed reaction is independent of pH under conditions where the catalytic constant varies with pH, it is equal to the thermodynamic dissociation constant of the enzyme–substrate complex. This is true for realistic mechanisms in which binding and catalytic steps are clearly distinguished, as well as for the simpler mechanisms that have been considered previously. It is also true for a mechanism in which a bell-shaped pH profile for the catalytic constant results from a change of rate-limiting step with pH. The relaxation time for ionization of a typical group in unbuffered solutions at 25°C is of the order of 0.1 ms at the longest, and is much shorter in buffered solutions. Thus ionizations in almost all enzyme mechanisms can properly be treated as equilibria, provided that ionization is not accompanied by a slow, compulsory change in conformation.

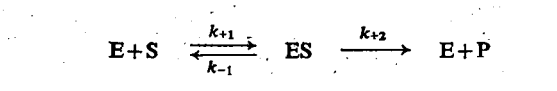
For many enzymes the initial velocity v, measured under steady-state conditions, is given by the Michaelis–Menten equation:

$$v = k_{cat}e_0s/(K_m+s) \quad (1)$$

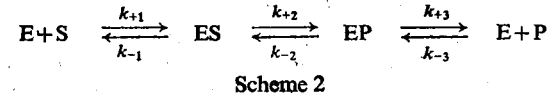
where e_0 is the total enzyme concentration, s is the free substrate concentration, k_{cat} is the catalytic constant, and K_m is the Michaelis constant. The Michaelis constant is the dissociation constant of the enzyme–substrate complex under steady-state conditions, but it is not a true thermodynamic constant, and it is often of less mechanistic interest than K_s, the equilibrium dissociation constant. K_s is not readily measurable, however, and much effort has been given to finding ways of estimating it and to determining circumstances in which K_m can be assumed to approximate to K_s.

For the two-step mechanism shown in Scheme 1 K_m is equal to $(k_{-1}+k_{+2})/k_{+1}$ (Briggs & Haldane, 1925), whereas K_s is equal to k_{-1}/k_{+1}. So the two are not equal unless $k_{-1} \gg k_{+2}$, as assumed by Michaelis & Menten (1913), and K_m can be as large as k_{+2}/k_{+1}, if $k_{-1} \ll k_{+2}$, as assumed by Van Slyke & Cullen (1914). In this two-step mechanism, therefore, K_m provides an upper limit for K_s, i.e. $K_s \leqslant K_m$, but this does not apply in more complex mechanisms (Dalziel, 1962), unless K_s is defined unconventionally. This is important, because Scheme 1 is not, in general, a plausible mechanism for enzyme catalysis, as discussed by Haldane (1930, pp. 80–83), and the simplest plausible mechanism is the three-step mechanism shown in Scheme 2. For this, K_m is given by $(k_{-1}k_{-2}+k_{-1}k_{+3}+k_{+2}k_{+3})/[k_{+1}(k_{-2}+k_{+2}+k_{+3})]$, which can be greater than, equal to, or less than K_s, if K_s is defined as k_{-1}/k_{+1}. It is arguable whether this is the most appropriate definition of K_s, however, and if the isomerization of ES to EP is regarded as a part of substrate binding it would be better to define K_s as the value of [E][S]/([ES] + [EP]) at equilibrium, i.e. $k_{-1}k_{-2}/[k_{+1}(k_{-2}+k_{+2})]$. This definition can readily be generalized to more complex cases, and it has the advantages that it avoids the need to make an arbitrary choice about where binding ends and catalysis begins, and it allows the limit $K_s \leqslant K_m$ to apply generally.

A widely applicable though rarely applied criterion of equality between K_m and K_s is one proposed originally by Haldane (1930, pp. 40–42), who noted that for yeast invertase the value of K_m is independent of pH in the range 4–8 whereas k_{cat} varies 20-fold. He argued that this could best be rationalized in terms of Scheme 1 if $k_{-1} \gg k_{+2}$, i.e. $K_s = K_m$. In general, the proposal is that any pure non-competitive effect (i.e. one that affects the apparent value of k_{cat} only), not necessarily one brought about by protons, provides evidence of equality between K_m and K_s. But, since pure non-competitive effects of species other than protons are rarely encountered except in textbooks,



Scheme 1



Scheme 2

the discussion in this paper will be in terms of protons. Haldane's argument has been discussed and supported more recently by Morales (1955) and Laidler (1958, pp. 117–130), albeit with the important qualification that other interpretations are possible, though they involve implausible assumptions about fortuitous proportionality between unrelated rate constants, or unaccountably zero rate constants. More recent discussions (e.g. Dixon & Webb, 1958; Webb, 1963; Gutfreund, 1965; Williams, 1969) have tended to ignore the qualifications, stating Haldane's conclusion as a simple fact.

In spite of its potential usefulness and the impressive range of authorities supporting it, the interpretation of pure non-competitive effects as evidence of equality between K_m and K_s has been largely ignored in the discussion of experimental data. For example, Denburg *et al.* (1968) observed only insignificant and unsystematic variation in K_m for the pepsin-catalysed hydrolysis of acetyl-L-phenylalanyl-L-tyrosine amide in the pH range 0.5–5.5, and were much concerned with estimating the value of K_s. But they did not relate the observation to the discussion; instead they considered the possible equality between K_m and K_s in terms of three weaker arguments.

The validity of Haldane's argument is not obvious, even for the simplest two-step mechanism, and its extension to more complex mechanisms has never been thoroughly discussed. Accordingly the purpose of this paper is to examine the interpretation of pH-independence of K_m in general, to determine whether it provides a valid argument for taking K_m as a measure of K_s.

Methods

All rate equations in this paper are derived by the method of King & Altman (1956), incorporating the suggestions of Cha (1968) for dealing with mechanisms containing steps that are assumed to be maintained at equilibrium. All ionization steps are treated as equilibria, an assumption that enormously simplifies the steady-state analysis of pH effects. For example, Scheme 5 (below) requires analysis of 384 patterns if the method of King & Altman (1956) is applied with no equilibrium assumptions, but the rate equation may be written down directly by Cha's (1968) method if the ionization steps can be treated as equilibria. Analytical convenience is not of course a sufficient justification for assuming ionization steps to be fast, and the validity of the assumption is discussed below.

Theory

Relaxation time for ionization

Ottolenghi (1971) has disputed the validity of the usual practice of treating ionization steps as equilibria in discussions of the pH dependence of enzymic catalysis. Taking a histidine residue with pK_a 7.0 as an example, and assuming that the rate constant for protonation by H_3O^+ is about $10^{10}M^{-1}\cdot s^{-1}$ (based on values given by Eigen & Hammes, 1963), one can calculate that the rate constant for deprotonation must be about $10^3 s^{-1}$, a value that is not necessarily large compared with other first-order rate constants in enzyme-catalysed reactions. But this calculation is unrealistic, because it assumes that water is the only significant acceptor of protons, whereas in fact OH^- is the principal acceptor in aqueous alkaline solutions, with rate constants of the order of $10^{10}M^{-1}\cdot s^{-1}$.

From the data of Eigen *et al.* (1960), one can calculate the relaxation time for ionization of imidazole at any pH, as follows. The rate constants for protonation by H_3O^+ and deprotonation by OH^- are both about $2.5\times10^{10}M^{-1}\cdot s^{-1}$ at 25°C, and the rate constants for protonation and deprotonation by H_2O are both about $2.5\times10^3 s^{-1}$; so the net rate of protonation is governed by a pseudo-first-order rate constant of about $2.5\times10^3(1+10^7[H^+])s^{-1}$, and the net rate of deprotonation is governed by a pseudo-first-order rate constant of about $2.5\times10^3(1+10^7[OH^-])s^{-1}$. The relaxation time τ is the reciprocal of the sum of these two values, i.e.

$$1/\tau = 5\times10^3\{1+\cosh[2.3(\mathrm{pH}-7)]\}s^{-1} \qquad (2)$$

Thus $1/\tau$ does not fall monotonically as the pH is raised, but instead has a minimum of $10^4 s^{-1}$ at pH 7. The relaxation time for protonation of a histidine residue in ribonuclease has been measured directly in unbuffered solution (Patel *et al.*, 1972), and is indeed of the order of 0.1 ms.

To the extent that imidazole can be taken as a model of an ionizing group in an enzyme, it is reasonable to treat ionization steps in most enzymic reactions as equilibria. This may require qualification if ionization is accompanied by a compulsory change in conformation (Fersht & Requena, 1971), though this may also be very rapid: Burke *et al.* (1965) observed relaxation times in the range 5×10^{-8} to 10^{-5} s for the helix–coil transition of poly-L-glutamate, though conformational changes for natural proteins are generally slower than this (see Hammes & Schimmel, 1970).

Pure non-competitive inhibition (or activation) by protons

A simple mechanism for non-competitive effects of protons is shown in Scheme 3. Charges are omitted to avoid confusion and because the difference in charge between protonated and unprotonated species is obvious in all cases. This mechanism was studied by Botts & Morales (1953), who derived the rate equation for the case where none of the steps could be treated as equilibria, and found it to be very complex. But if the ionization steps are treated as equilibria the

initial rate for this mechanism is given by eqn. (1), with k_{cat} and K_m defined as follows:

$$k_{cat} = \frac{k_{+2} + k'_{+2}h/K'_H}{1 + h/K'_H} \quad (3)$$

$$K_m = \frac{[k_{-1} + k_{+2} + (k'_{-1} + k'_{+2})h/K'_H](1 + h/K_H)}{(k_{+1} + k'_{+1}h/K_H)(1 + h/K'_H)} \quad (4)$$

where h is the proton activity, i.e. 10^{-pH}.

It is clear from eqn. (3) that k_{cat} can vary with pH only if k'_{+2} differs from k_{+2}. In practice the lower limit for k_{cat} is usually zero, and so it is appropriate to put $k'_{+2} = 0$. This implies inhibition by protons, but activation can readily be produced if k_{+2} is zero instead of k'_{+2}. This gives analogous equations, and will not be discussed separately.

Although it is possible for K_H to differ from K'_H and yet for K_m to be independent of pH, if $k_{+1}/k'_{+1} = k'_{-1}/(k_{-1} + k_{+2}) = 1 \simeq k'_{-1}/k_{+2}$, this relationship is too implausible to be considered as a general case, and in the remainder of this paper K'_H will be assumed to be the same as K_H. This restriction is commonly regarded as an essential characteristic of pure non-competitive inhibition (see, e.g., Laidler, 1958, pp. 78–80), and is in no way a special assumption of this paper. Another relationship between the rate constants results from the thermodynamic requirement for the equilibrium constant of any reaction to be independent of pathway. This means that $k'_{-1}/k'_{+1}K'_H = k_{-1}/k_{+1}K_H$, i.e. $k'_{-1}/k'_{+1} = k_{-1}/k_{+1}$ if $K'_H = K_H$.

Equations (3) and (4) are greatly simplified by these relationships, and become

$$k_{cat} = k_{+2}/(1 + h/K_H) \quad (5)$$

$$K_m = \frac{k_{-1}}{k_{+1}}\left(\frac{k_{-1} + k_{+2} + k'_{-1}h/K_H}{k_{-1} + k'_{-1}h/K_H}\right) \quad (6)$$

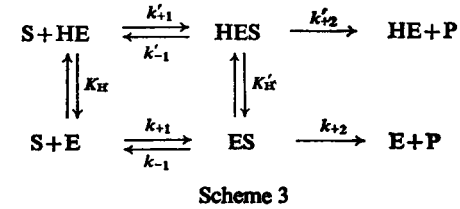
Pure non-competitive effects are characterized experimentally by variation in k_{cat} without variation in K_m. But according to eqns. (5) and (6) both k_{cat} and K_m are functions of pH, and it is necessary therefore to consider the circumstances in which K_m can be independent of pH without violating eqn. (6). There are two such circumstances, namely (i) $k_{+2} \ll k_{-1}$, and (ii) $k'_{-1}h \ll k_{-1}K_H$ over the whole range of pH considered. Laidler (1958, pp. 117–130) mentioned a third possibility, that certain rate constants are equal by coincidence, but this does not apply if, as here, ionization steps are assumed to be equilibria. Laidler (1958, pp. 117–130) considered that the second possibility was unreasonable for protons, since if protonation has no effect on the equilibrium constant for substrate binding it is difficult to see how it can affect the rate constants sufficiently to render K_m independent of pH. Instead it is likely that $k'_{-1} \simeq k_{-1}$. Thus he concluded that in most instances $k_{+2} \ll k_{-1}$ was the only reasonable interpretation of pure non-competitive effects.

It follows then that if Scheme 3 is used as a model, pure non-competitive effects do indeed demonstrate the identity of K_m and K_s. Unfortunately, however, Scheme 3 is neither the only nor the most reasonable mechanism for pure non-competitive effects. (It has been considered at this length because it is the principal model discussed by previous authors.) Scheme 3 is in fact seriously defective, in that it makes no proper distinction between binding steps and catalytic steps. It is scarcely reasonable to treat substrate binding as a simple reaction, but product release as a fundamentally different type of reaction involving a chemical change. In many biochemical reactions substrate and product are analogues, and equilibrium constants are often close to unity, and it is absurd to suppose that protonation has no effect on substrate binding but has drastic effects on product release. This absurdity can be resolved by supposing that there is at least one chemical step between the binding of substrate and the release of product, in other words by proposing Scheme 4, which bears the same relationship to Scheme 3 as Scheme 2 does to Scheme 1. With this mechanism the effect of the proton can be accounted for much more plausibly, by supposing that it plays an essential catalytic role in the conversion of HES into HEP, or that the conjugate base is essential for the conversion of ES into EP. In the first case k_{-2} and k_{+2} would be zero; in the second k'_{-2} and k'_{+2} would be zero.

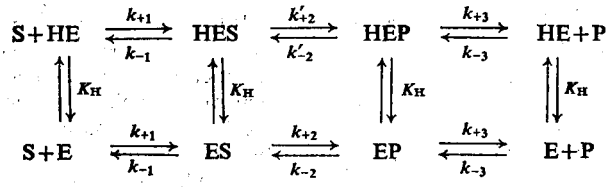
Scheme 4 is a special case of the rather more important model to be considered next, and all rate equations required for discussing it can readily be derived by omitting terms from the equations to be given. To avoid unnecessary repetition of similar algebra, therefore, Scheme 4 will not be discussed further.

Bell-shaped pH-dependence of k_{cat}

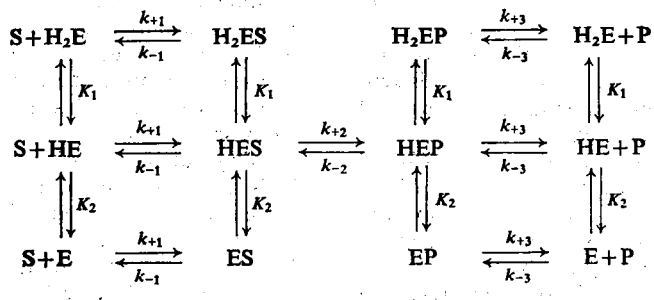
It is often observed that the pH profile of k_{cat} is bell-shaped, i.e. it approaches zero asymptotically at both high and low pH values, but is finite at intermediate values. The theory of this behaviour has been developed by Michaelis & Davidsohn (1911), Michaelis (1922), Waley (1953), Alberty & Massey (1954) and Dixon (1973). It is commonly interpreted to mean that there are two specific ionizable groups



Scheme 3



Scheme 4



Scheme 5

on the enzyme, of which one must be protonated and the other deprotonated for catalysis to be possible. This is shown, for a three-step mechanism, in Scheme 5, which is the same as Scheme 2 with all six rate constants made pH-dependent. The initial rate in the absence of added product is given by eqn. (1), with k_{cat} and K_m defined as follows:

$$k_{cat} = \frac{k_{+2}k_{+3}f(h)}{(k_{-2}+k_{+2})f(h)+k_{+3}} \quad (7)$$

$$K_m = \frac{1}{k_{+1}}\left[k_{-1}+\frac{k_{+2}(k_{+3}-k_{-1})f(h)}{(k_{-2}+k_{+2})f(h)+k_{+3}}\right] \quad (8)$$

where $f(h)$, a function given by Michaelis (1922), is defined by

$$f(h) = 1/[(h/K_1)+1+(K_2/h)] \quad (9)$$

For analytical purposes it is sometimes more convenient to express the Michaelis function directly in terms of pH, which may be done as follows (cf. Dixon, 1974):

$$f(h) = K_1^{\frac{1}{2}}/\{K_1^{\frac{1}{2}}+2K_2^{\frac{1}{2}}\cosh[2.303(\mathrm{pH}-\mathrm{pH_{opt}})]\} \quad (10)$$

where pH_{opt} is the pH value at which $f(h)$ is a maximum, i.e. $pH_{opt} = \frac{1}{2}(pK_1 + pK_2)$.

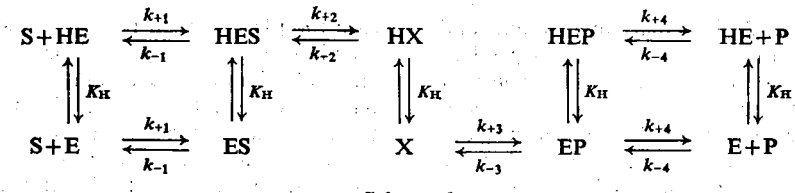
According to eqn. (7) k_{cat} displays a bell-shaped pH profile and approaches zero at both extremes. The shape of the pH profile of K_m is either a bell (if $k_{+3} > k_{-1}$) or an inverted bell (if $k_{+3} < k_{-1}$), with non-zero asymptotes, both equal to k_{-1}/k_{+1}. K_m can be independent of pH either if k_{+2} is small, or if $k_{-1} = k_{+3}$. In either case K_m is then equal to k_{-1}/k_{+1}, the equilibrium constant for the first step, at all pH values.

The possibility that $k_{-1} = k_{+3}$ is not simply an unlikely coincidence that can safely be dismissed from serious consideration in most instances. Many enzyme-catalysed reactions involve minor changes in large molecules. In such reactions the groups involved in binding may be virtually identical for substrate and product, and the rate constants for substrate and product release may then be equal. The relationship $K_m = k_{-1}/k_{+1}$ also applies in the corresponding pH-independent mechanism, Scheme 2, if $k_{-1} = k_{+3}$, regardless of the relative values of k_{-1} and k_{+2}.

Both of the possible circumstances that make K_m pH-independent give the same value for K_m, namely k_{-1}/k_{+1}. So it may be argued that it is of little consequence which interpretation is correct. But there are two reasons why the question should be considered. The fact that K_m is equal to k_{-1}/k_{+1} cannot be taken as conclusive evidence that k_{+2} is small, and that the interconversion of HES and HEP is rate-limiting, as this need not be true if $k_{-1} = k_{+3}$. Moreover, the existence of two possibilities has important implications for the interpretation of the pH-dependence of k_{cat}. It is common practice to explain bell-shaped pH profiles in terms of an equation of the form

$$k_{cat} = \bar{k}_{cat}/[(h/K'_1)+1+(K'_2/h)] \quad (11)$$

where $\bar{k}_{cat}$ is the 'pH-corrected' parameter, i.e. the value that k_{cat} would have if all enzyme molecules



Scheme 6

were in the reactive ionic state. But although eqns. (7) and (11) define a similar range of curves they are not directly comparable, and the experimentally measurable quantities K_1' and K_2' are not equal to the mechanistically interesting quantities K_1 and K_2. Instead K_1' is larger than K_1 and K_2' is smaller than K_2, since

$$K_1'/K_1 = K_2/K_2' = 1+(k_{+2}/k_{+3})+(k_{-2}/k_{+3}) \quad (12)$$

Thus the empirical pK values, pK_1' and pK_2', are further apart than the true pK values, pK_1 and pK_2. This type of behaviour is common for pH profiles in other situations (Brocklehurst & Dixon, 1976). The position of the pH optimum, given by $\frac{1}{2}(pK_1'+pK_2')$, is unaffected, because $K_1'K_2' = K_1K_2$. The importance of this analysis is that it shows that perturbation of pK values for k_{cat} is possible even if K_m is independent of pH.

Changes of rate-limiting step with pH

Jencks (1969) has extensively discussed the possibility that the pH-dependence of reaction rates can result from changes in rate-limiting step with pH. Dixon (1973) has applied these ideas to bell-shaped pH profiles, though not explicitly in enzyme catalysis. A simple mechanism for an enzyme-catalysed reaction is shown in Scheme 6, requiring only a single ionizing group, which must be protonated in the second step and deprotonated in the third. As in Scheme 5, protonation is assumed to have no effect on either substrate or product binding. The rate is given by eqn. (1), with k_{cat} and K_m defined as follows:

$$k_{cat} = \frac{k_{+2}k_{+3}k_{+4}}{(k_{-2}+k_{+2})k_{+4}(h/K_H)+k_{-2}k_{-3}+k_{-2}k_{+4}+k_{+2}k_{-3}+k_{+2}k_{+3}+k_{+2}k_{+4}+k_{+3}k_{+4}+k_{+3}k_{+4}(K_H/h)} \quad (13)$$

$$K_m = \frac{k_{-1}k_{-2}k_{+4}(h/K_H)+k_{-1}k_{-2}k_{-3}+k_{-1}k_{-2}k_{+4}+k_{-1}k_{+3}k_{+4}+k_{+2}k_{+3}k_{+4}+k_{-1}k_{+3}k_{+4}(K_H/h)}{k_{+1}[(k_{-2}+k_{+2})k_{+4}(h/K_H)+k_{-2}k_{-3}+k_{-2}k_{+4}+k_{+2}k_{-3}+k_{+2}k_{+3}+k_{+2}k_{+4}+k_{+3}k_{+4}+k_{+3}k_{+4}(K_H/h)]} \quad (14)$$

Eqn. (13) is of the same form as eqn. (7), and leads to the similar conclusion that experimental pK values are not the same as pK_H. In addition they are unsymmetrically displaced from pK_H, because h/K_H and K_H/h have different coefficients in eqn. (13). So the pH at which k_{cat} is a maximum is not pK_H, but $pK_H + \frac{1}{2}\log[(k_{-2}+k_{+2})/k_{+3}]$, the perturbation term being negligible only in the unlikely event that $k_{+3} \simeq k_{-2}+k_{+2}$.

Eqn. (14) differs from eqn. (8) in that it defines an unsymmetrical pH profile for K_m, with different limits at the two extremes, i.e. $K_m \to k_{-1}k_{-2}/[k_{+1}(k_{-2}+k_{+2})]$ as $h \to \infty$ (low pH) and $K_m \to k_{-1}/k_{+1}$ as $h \to 0$ (high pH). But this is unlikely to be diagnostically useful, because any variation of K_m with pH would argue against the initial assumption that the acid dissociation constants in either mechanism are unaffected by substrate or product binding.

For Scheme 6, the limiting values of K_m are equal only if $k_{+2} \ll k_{-2}$, and then both are equal to k_{-1}/k_{+1}, the equilibrium constant of the first step. K_m is independent of pH and equal to k_{-1}/k_{+1} at all pH values either if $k_{+2} \ll k_{-1}$, k_{+4}, or if $k_{-1} = k_{+4}$.

It is arguable that, although it may be reasonable to assume that HE, HES and HEP have the same acid dissociation constant K_H, it is by no means equally reasonable to assign the same constant to HX, enclosed as it is by pH-dependent reactions. If a different acid dissociation constant K_H' is assumed for HX, the analysis of Scheme 6 becomes more complicated, because about half of the terms in eqns. (13) and (14), namely those that contain k_{-2} or $(k_{-2}+k_{+2})$ as a factor, must then be multiplied by K_H/K_H', and the term $k_{+2}k_{-3}$ in the denominators of both expressions must be replaced by the pH-dependent term $k_{+2}k_{-3}(1+h/K_H')/(1+h/K_H)$. However, the interpretation of the pH-independence of K_m requires only slight modification, with the extra requirement that $k_{+2} \ll k_{-2}K_H/K_H'$. Again, if K_m is pH-independent it must be equal to k_{-1}/k_{+1}.

Discussion

In spite of the variety and complexity of the mechanisms for pH effects that have been examined in this

paper, the main result is remarkably simple and general, confirming that K_m can only be independent of pH if it is equal to the equilibrium constant k_{-1}/k_{+1} of the first step of the reaction. This was of course known already for simple mechanisms, but the fact that it applies also to mechanisms that are complex enough to be realistic is likely to be of considerable practical value in interpreting kinetic results. On the other hand, the interpretation of the pH-dependence of k_{cat}, even when it is uncomplicated by a concomitant variation of K_m, is more complex than has usually been realized, and it is clear that assignment of group dissociation constants on the basis of empirical pK values is a dangerous procedure. Provided the dangers are realized and that assignments are supported by other evidence, however, the simple interpretation is likely to prove of continuing value as a working hypothesis. If no naive interpretations of pH profiles had ever been permitted many important advances in the understanding of enzymic catalysis would have been prevented. Indeed it is difficult to find an example where dissociating groups deduced from observed pK values proved subsequently to be seriously misleading.

The assumption made in this paper that ionizations can be treated as equilibria may prove to be false in some instances, though there are few enzymes that have k_{cat} values greater than the $10^4 s^{-1}$ calculated as the minimum value of $1/\tau$ for ionization of imidazole at 25°C. Since the calculation was made without any allowance for buffer effects it is likely that ionizations in buffered solutions would be much faster. Indeed, if non-equilibrium ionizations are suspected, measurement of the dependence of the kinetics on buffer strength should provide a useful test: at a concentration of 0.1 M an acid with a rate constant of $10^9 M^{-1} \cdot s^{-1}$ for protonation of imidazole will react more than four orders of magnitude faster than water at 56M. Buffer effects of this type have been observed in carbonic anhydrase (Silverman & Tu, 1975), but this is an example of very fast catalysis rather than of slow ionization.

The results in this paper lead to a conjecture that, if correct, may also prove valuable in the interpretation of pH effects. In all of the mechanisms examined, the limiting values of K_m at high and low pH were either k_{-1}/k_{+1} or some other equilibrium constant. {For Scheme 6, the limit at low pH was $k_{-1}k_{-2}/[k_{+1}(k_{-2}+k_{+2})]$, which is the equilibrium constant for dissociation of S from the composite species HES + HX.} Inspection of the mechanisms suggests that even though this conclusion may require modification if the acid dissociation constants are different for each intermediate a correspondingly useful generalization may be possible.

One possibility not considered in this paper is that there may be two or more steps in a reaction that require the enzyme in the same ionic state. In such a case the pH effects in the various steps may cancel fortuitously to give a pH-independent K_m that is not an equilibrium constant (Bauer & Pettersson, 1974), but this seems unlikely to be of common occurrence. If such fortuitous cancelling is suspected it may be appropriate to study the effect of pH on K_m at several temperatures, as it would be difficult to argue that such a coincidence would occur at every temperature.

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