pH-Dependence of the Steady-State Rate of a Two-Step Enzymic Reaction

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1. The pH-dependence is considered of a reaction between E and S that proceeds through an intermediate ES under 'Briggs-Haldane' conditions, i.e. there is a steady state in ES and $[S]_0 \gg [E]_T$, where $[S]_0$ is the initial concentration of S and $[E]_T$ is the total concentration of all forms of E. Reactants and intermediates are assumed to interconvert in three protonic states ($E \rightleftharpoons ES$; $EH \rightleftharpoons EHS$; $EH_2 \rightleftharpoons EH_2S$), but only EHS provides products by an irreversible reaction whose rate constant is $k_{cat.}$. Protonations are assumed to be so fast that they are all at equilibrium. 2. The rate equation for this model is shown to be $v = d[P]/dt = (k_{cat.}[E]_T[S]_0/A)/[(\tilde{K}_m BC/DA) + [S]_0]$, where $\tilde{K}_m$ is the usual assembly of rate constants around EHS and A–D are functions of the form $(1 + [H]/K_1 + K_2/[H])$, in which K_1 and K_2 are: in A, the molecular ionization constants of ES; in B, the analogous constants of E; in C and D, apparent ionization constants composed of molecular ionization constants (of E or ES) and assemblies of rate constants. 3. As in earlier treatments of this type of reaction which involve either the assumption that the reactants and intermediate are in equilibrium or the assumption of Peller & Alberty [(1959) *J. Am. Chem. Soc.* **81**, 5907–5914] that only EH and EHS interconvert directly, the pH-dependence of $k_{cat.}$ is determined only by A. 4. The pH-dependence of K_m is determined in general by $B \cdot C/A \cdot D$, but when reactants and intermediate are in equilibrium, $C \equiv D$ and this expression simplifies to B/A . 5. The pH-dependence of $k_{cat.}/K_m$, i.e. of the rate when $[S]_0 \ll K_m$, is not necessarily a simple bell-shaped curve characterized only by the ionization constants of B, but is a complex curve characterized by $D/B \cdot C$. 6. Various situations are discussed in which the pH-dependence of $k_{cat.}/K_m$ is determined by assemblies simpler than $D/B \cdot C$. The special situation in which a $k_{cat.}/K_m$ -pH profile provides the molecular pK_a values of the intermediate ES complex is delineated.

One way to characterize a chemical reaction in aqueous media is to study the pH-dependence of its kinetic parameters. This is sometimes done as part of an attempt to locate protons in transition states and sometimes, perhaps more realistically, to assist in the characterization of the environments of ionizing groups in the reactant molecules (see, e.g., Brocklehurst, 1974). A situation that has been deeply explored by many workers (see H. Dixon, 1973) is the one commonly assumed to apply in enzyme reactions in which loss or gain of one proton from a particular ionization state renders the system unreactive.

There exists considerable literature on pH-rate profiles and the problems inherent in their interpretation. Widely recognized aspects of these problems include (1) perturbations of kinetically determined pK_a values by general microenvironmental solvation effects (see, e.g., Schmidt & Westheimer, 1971), by specific interactions between proton-binding sites (see, e.g., H. Dixon & Tipton, 1973; Brocklehurst, 1974; Polgar, 1974; Shipton *et al.*, 1975) and by pre-equilibria (Bruce & Schmir, 1959; Caplow, 1969)

and (2) production of mirage pK_a values by change in rate-limiting step with pH (Jencks, 1959, 1969; H. Dixon, 1973). The concept of transition-state acid dissociation constants introduced by Kurz (1963, 1972) has been applied to the study of enzyme reactions by Dunford (see, e.g., Dunford *et al.*, 1974).

One approach that is often used in enzyme characterization is the determination of pH-rate profiles at 'low' (non-saturating) concentrations of substrate (S) or group-specific reagent (R), such that the reaction is first-order in S or R. Under these conditions it is usually assumed that the pH-rate profile provides pK_a values that characterize the free reactant state (enzyme and/or substrate or reagent). This question, however, has not been fully discussed in terms of a reasonably general rate equation. The exact steady-state treatment of a completely general two-step model leads to an equation of great complexity (Laidler, 1955), which cannot be applied to experimental data. Restriction of the model by certain simplifying assumptions does indeed lead to a rate equation that predicts pH-rate profiles characterized

by free-reactant-state pK_a values. Perhaps the most useful of these restrictions is the assumption of an equilibrium situation for all reversible steps instead of a steady state (Laidler, 1955). Even if the steady-state assumption is retained, free-reactant-state pK_a values are still predicted if it is assumed that direct interconversion of enzyme and enzyme-substrate complex occurs only in one ionization state of the system (Peller & Alberty, 1959). This is essentially one of the several simplifying assumptions made by Laidler (1955). The severe restrictions imposed by the somewhat implausible assumption of Peller & Alberty (1959) were widely accepted or ignored until attention was drawn to them by Schmidt & Westheimer (1971) and by Plaut & Knowles (1972).

The present paper reports the derivation of a reasonably general steady-state rate equation for a two-step reaction, in which only the ionizations are regarded as unperturbed equilibria. The equation is used to delineate situations in which kinetically determined parameters have simple significance, and to predict shapes of pH-rate profiles.

The rate equation derived in the present paper predicts the pH-rate profiles obtained in non-equilibrium steady-state situations at low non-saturating substrate concentrations. Such profiles need not be symmetrical bell-shaped curves, and will in general provide apparent pK_a values composed of free-reactant dissociation constants, enzyme-substrate-complex dissociation constants and assemblies of the other rate constants of the kinetic model. In some cases in which a bell-shaped component appears in the profile, the two apparent pK_a values that characterize it can arise not from different ionizations but from the same one; one of the apparent pK_a values is shifted from the true pK_a value by a factor containing rate constants.

When some of the rate constants bear rather special relationships to each other such profiles can provide the molecular pK_a values of the enzyme-substrate (or reagent) complex rather than those of free enzyme as is usually assumed. The possibility that dissociation constants of an enzyme-reagent complex may be expressed in profiles of $k_{cat.}/K_m$ against pH was first pointed out by J. R. Knowles (unpublished work, described at a 1972 meeting of the Biochemical Society Molecular Enzymology Group, London, U.K.).

Derivation of the Rate Equation

(1) The model

A two-step reaction in which reactants E and S react through the intermediacy of an ES complex or compound is considered (e.g. Scheme 1). Reactant and intermediate states are assumed to interconvert through three protonic states, but only one protonic state of the intermediate (EHS) is assumed to provide

Scheme 1. Representation of a two-step reaction involving the reversible formation of an intermediate in three protonic states followed by an irreversible step involving only one protonic state

$k_{+1}-k_{+3}$, $k_{-1}-k_{-3}$ and $\tilde{k}_{cat.}$ are pH-independent rate constants and K_{eI} , K_{eII} , K_{esi} and K_{esII} are molecular acid dissociation constants; 'free' protons are omitted for clarity. The reactants could be an enzyme E and a substrate or group-specific reagent S. When a complete catalysis is considered, the products, P, will include the regenerated enzyme E.

products, in an irreversible step. For convenience, the ionizations of the reactant state are formally associated with one reactant E.

Systems of interest would include those in which E is an enzyme and S is a substrate or group-specific reagent. When the decomposition of EHS regenerates E, Scheme 1 represents a complete catalysis. If E is not regenerated, Scheme 1 could represent a partial catalysis (e.g. formation of an acyl-enzyme) or reaction of a particular group in the enzyme with a group-specific reagent. The present discussion is presented in terms of S and $\tilde{k}_{cat.}$, but when a complete catalysis is not considered, one or both of these will not have their usual significance.

One restriction of the model chosen should be noted; it does not cover cases in which EHS forms an enzyme-product complex, whose steady-state concentration is appreciable in comparison with the forms considered. The pH-dependence of K_m for such extended models has been treated by Cornish-Bowden (1976). When complete catalyses are being considered, this largely restricts the model to enzymes in which the chemical conversion is relatively slow, and this may not be as common as is often supposed (J. Südi, personal communication; see also Hansen *et al.*, 1969). This difficulty does not, of course, obtain when the treatment is being applied to partial catalyses or to reactions with group-specific reagents.

(2) The assumptions

The reactions of Scheme 1 are assumed to involve rapidly established vertical equilibria (ionizations) and a horizontal steady state.

(a) *Ionizations as equilibria.* The usual practice of treating ionizations as equilibria has been criticized by Ottolenghi (1971). He considered an acid with dissociation constant $K_a = k_{-a}/k_{+a} = 10^{-7} \text{ M}$, and argued that because the rate constant for protonation (k_{+a}) should be approx. $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$ (Eigen & Hammes, 1963) the rate constant for deprotonation (k_{-a}) should be approx. $10^{-7} \times 10^{10} = 10^3 \text{ s}^{-1}$, which is similar in magnitude to some other rate constants found in enzyme catalysis (Eigen & Hammes, 1963).

If this argument were valid, analytical study of the pH-dependence of enzyme kinetic parameters would be virtually impossible. Cornish-Bowden (1976), however, has pointed out that Ottolenghi's (1971) calculation is unrealistic because it assumes that water is the only significant acceptor of protons, whereas, in fact, HO^- is the principal acceptor in aqueous alkaline solutions, and HO^- reactions (with rate constant approx. $10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}$) could be of considerable significance also in neutral and weakly acidic media (see also Jencks, 1969, pp. 207–211).

The relaxation time for the ionization of an acid group, $pK_a = 7$, is likely to be less than 0.1 ms at 25°C (and much shorter in buffered solutions). This limit suggests that ionizations may be treated as equilibria in many enzyme mechanisms (Cornish-Bowden, 1976). E. M. Crook (personal communication) has pointed out to us that this conclusion may be limited to concentrations of catalyst that do not exceed $[\text{HO}^-]$, else the rate of production of HO^- from water could limit the rate of equilibration of a group by the HO^- pathway.

(b) *The steady-state assumption.* W. W. Cleland (quoted in Mahler & Cordes, 1966, p. 226) has demonstrated that, for a two-step catalytic reaction of the type depicted by the reaction sequence $\text{EH} + \text{S} \rightleftharpoons \text{EHS} \rightarrow \text{EH} + \text{P}$ of Scheme 1 with the rate constants approximately equal and $[\text{EH}]_0 \approx [\text{S}]_0$, the steady-state assumption is valid. He has demonstrated also (quoted in Mahler & Cordes, 1966, p. 224) that for a similar, but non-catalytic, reaction sequence, i.e. one in which E is not regenerated, the steady state exists for only a small fraction of the reaction, if relationships similar to those given above obtain between the three rate constants and the two initial concentrations. If, however, the reaction is studied with one reactant in considerable excess and if the intermediate does not accumulate to an appreciable extent, the steady-state assumption should be valid (see Jencks, 1969, pp. 590–591).

(3) Definitions and derivation

The acid dissociation constants that characterize Scheme 1 are defined by eqn. (1)–(4); charges are omitted throughout, e.g. $[\text{H}] = [\text{H}^+]$. The conservation of enzyme is expressed in eqn. (5), in which

$[\text{E}]_T$ = total enzyme concentration, and the rate equation is eqn. (6). It is assumed that $[\text{S}]_0$, the initial concentration of S, is much greater than $[\text{E}]_T$, and P is negligible, i.e. initial rates (v_i) are measured.

$$K_{\text{el}} = [\text{EH}][\text{H}]/[\text{EH}_2] \quad (1)$$

$$K_{\text{eII}} = [\text{E}][\text{H}]/[\text{EH}] \quad (2)$$

$$K_{\text{esI}} = [\text{EHS}][\text{H}]/[\text{EH}_2\text{S}] \quad (3)$$

$$K_{\text{esII}} = [\text{ES}][\text{H}]/[\text{EHS}] \quad (4)$$

$$[\text{E}]_T = [\text{E}] + [\text{EH}] + [\text{EH}_2] + [\text{ES}] + [\text{EHS}] + [\text{EH}_2\text{S}] \quad (5)$$

$$v_i = d[\text{P}]/dt = \tilde{k}_{\text{cat}}[\text{EHS}] \quad (6)$$

Application of the conventional steady-state treatment to the fluxes around ES, EHS and EH_2S (see Scheme 1) by using eqns. (5) and (6) provides eqn. (7), in which the various factors are defined by eqns. (8)–(12). Such treatment is facilitated by giving rate constants to the reactions between EH_2S , EHS and ES. Eqn. (7) can be simply derived also by the method of Cha (1968).

$$\frac{d[\text{P}]}{dt} = \frac{\tilde{k}_{\text{cat}} [\text{E}]_T [\text{S}]_0}{\tilde{K}_m B \cdot C \over D \cdot A + [\text{S}]_0} \quad (7)$$

$$A = \left(1 + \frac{[\text{H}]}{K_{\text{esI}}} + \frac{K_{\text{esII}}}{[\text{H}]} \right) \quad (8)$$

$$B = \left(1 + \frac{[\text{H}]}{K_{\text{el}}} + \frac{K_{\text{eII}}}{[\text{H}]} \right) \quad (9)$$

$$C = \left[1 + \frac{[\text{H}]}{K_{\text{esI}}} \left(\frac{k_{-1}}{k_{-2} + \tilde{k}_{\text{cat}}} \right) + \frac{K_{\text{esII}}}{[\text{H}]} \left(\frac{k_{-3}}{k_{-2} + \tilde{k}_{\text{cat}}} \right) \right] \quad (10)$$

$$D = \left(1 + \frac{[\text{H}]}{K_{\text{el}}} \cdot \frac{k_{+1}}{k_{+2}} + \frac{K_{\text{eII}}}{[\text{H}]} \cdot \frac{k_{+3}}{k_{+2}} \right) \quad (11)$$

$$\tilde{K}_m = (k_{-2} + \tilde{k}_{\text{cat}})/k_{+2} \quad (12)$$

The well-known rate equation for a reaction of the type shown in Scheme 1, written in terms of pH-dependent parameters k_{cat} , and K_m , is eqn. (13), and the pH-dependent parameters of eqn. (13) are related to the pH-independent parameters of eqn. (7) by eqns. (14)–(16).

$$\frac{d[\text{P}]}{dt} = \frac{k_{\text{cat}} [\text{E}]_T [\text{S}]_0}{K_m + [\text{S}]_0} \quad (13)$$

$$k_{\text{cat}} = \tilde{k}_{\text{cat}}/A \quad (14)$$

$$K_m = \tilde{K}_m \cdot \frac{B \cdot C}{D \cdot A} \quad (15)$$

$$k_{\text{cat}}/K_m = \frac{\tilde{k}_{\text{cat}}}{\tilde{K}_m} \cdot \frac{D}{B \cdot C} \quad (16)$$

The relationship between factors C and D. Not all the characterizing parameters of Scheme 1 are independent of each other. The equilibrium constant for the dissociation of EHS into E, H and S must be the same whether the process occurs via ES or via EH, and a similar argument applies to the dissociation of EH₂S into EH, H and S. The interdependence of the parameters is expressed by eqns. (17) and (18), and one consequence of this is that the expression for D (eqn. 11) may be written alternatively as eqn. (19).

$$K_{\text{esII}} \cdot \frac{k_{-3}}{k_{-2}} = K_{\text{eII}} \cdot \frac{k_{+3}}{k_{+2}} \quad (17)$$

$$K_{\text{esI}} \cdot \frac{k_{-2}}{k_{-1}} = K_{\text{eI}} \cdot \frac{k_{+2}}{k_{+1}} \quad (18)$$

$$D = \left(1 + \frac{[\text{H}]}{K_{\text{esI}}} \cdot \frac{k_{-1}}{k_{-2}} + \frac{K_{\text{esII}}}{[\text{H}]} \cdot \frac{k_{-3}}{k_{-2}} \right) \quad (19)$$

Comparison of eqns. (10) and (19) shows that if $k_{-2} \gg \bar{k}_{\text{cat.}}$, which is a sufficient but not necessary condition for equilibrium to be established between E, EH, EH₂, ES, EHS and EH₂S [see the Discussion, section 3(d)II], then C becomes identical with D. The terms C and D therefore cancel in the expression (eqn. 16) for $k_{\text{cat.}}/K_m$.

Discussion

(1) The pH-dependence of $k_{\text{cat.}}$

Eqn. (14) predicts, necessarily in agreement with earlier considerations of restricted versions of Scheme 1, that study of the pH-dependence of $k_{\text{cat.}}$ will provide the acid dissociation constants of the ES complex, K_{esI} and K_{esII} .

If there are several interconvertible forms of the ES complex on the reaction pathway, and these have different values of K_{esI} and K_{esII} , the values provided are averages of those for the various forms, weighted in favour of the predominant forms. This may be shown in a way such as that used by Fastrez & Fersht (1973) for the pH-dependence of K_m when non-productive binding accompanies productive binding. Thus if an enzyme E combines with S to form ES, whose protonated form ESH has an acid dissociation constant K , and if ES is transformed into ES', whose protonated form ES'H has an acid dissociation constant K' , and if only ES' is transformed into products, the value of the acid dissociation constant that appears in $k_{\text{cat.}}$ is $(1 + \alpha)KK'/(K + \alpha K')$, where α is the steady-state ratio of ES to ES' (i.e. the ratio between the unprotonated forms). Hence if α is so large that $\alpha \gg 1$ and $\alpha K' \gg K$, this constant becomes K , and when α is so small that $\alpha \ll 1$ and $\alpha K' \ll K$, the constant becomes K' ; thus $k_{\text{cat.}}$ shows the $\text{p}K_a$ values of the predominating species of ES. If, in addition, the forms ESH and ES'H are interconvertible, then,

unless equilibrium obtains, the ratio between the unprotonated forms of ES and ES' itself varies with pH; in that case the limb of the $k_{\text{cat.}}$ -pH profile considered cannot be described by a single value of K .

(2) The pH-dependence of K_m

Eqn. (15) shows that the pH-dependence of K_m is complex and depends on all four acid dissociation constants (K_{eI} , K_{eII} , K_{esI} and K_{esII}) and assemblies of all the other rate constants of Scheme 1. Even when the equilibrium situation obtains, the pH-dependence of K_m is still complex. In this situation, $C \equiv D$ and, as is well known, the pH-dependence of K_m depends on the ratio B/A (M. Dixon, 1953).

(3) The pH-dependence of $k_{\text{cat.}}/K_m$

(a) *General.* The main purpose of this paper is the consideration of the pH-dependence of $k_{\text{cat.}}/K_m$. This ratio is a useful specificity index (as emphasized in unpublished work by J. Südi), which obviates problems of non-productive binding (Bender & Kézdy, 1965; Brocklehurst *et al.*, 1968). For a discussion of its value as an index of enzyme efficiency in equilibrium situations, see Fersht (1974). The fact that only three of the factors A–D appear in the expression for $k_{\text{cat.}}/K_m$ (eqn. 16), but all four appear in the expression for K_m (eqn. 15), means that the analysis of its pH-dependence is simpler. Further, $k_{\text{cat.}}/K_m$ can be determined even if observations are limited (e.g. by substrate solubility; see Cornish-Bowden & Knowles, 1969) to conditions where $[\text{S}]_0 \ll K_m$, when the reaction is first-order in S (see eqns. 7 and 13), and $k_{\text{cat.}}$ and K_m cannot be separately determined.

Eqn. (16) shows that even when the ionizations of Scheme 1 are assumed to be unperturbed equilibria, the pH-dependence of $k_{\text{cat.}}/K_m$ in the otherwise general steady-state case does not provide simple acid dissociation constants. One result of this analysis of Scheme 1 is that eqn. (16) suggests that the pH-dependence of $k_{\text{cat.}}/K_m$ need not conform to a symmetrical bell-shaped curve (see below).

(b) *Particular situations.* (I) Situations in which the Peller & Alberty (1959) assumption applies. In these cases, EH and EHS are the only interconvertible forms of reactant and intermediate complex states, and eqn. (20) applies.

$$k_{+1} = k_{-1} = k_{+3} = k_{-3} = 0 \quad (20)$$

In such cases, both C and D of eqn. (16) reduce to unity, and eqn. (16) then predicts that the pH-dependence of $k_{\text{cat.}}/K_m$ will take a symmetrical bell-shaped form, and will provide K_{eI} and K_{eII} , the free-reactant ionization constants.

(II) Situations in which the equilibrium assumption applies. It is well known that, when a rate equation is

derived for Scheme 1 by making the assumption that all the reversible steps are at equilibrium, it predicts that the pH-dependence of $k_{\text{cat.}}/K_m$ will be of symmetrical bell-shaped form and will provide K_{el} and K_{eil} , just as when the Peller & Alberty (1959) assumption applies. It was demonstrated above that the equilibrium assumption leads to identity of C and D (see eqns. 10 and 19). The consequence of this for eqn. (16) is that the pH-dependence of $k_{\text{cat.}}/K_m$ in this situation depends only on B, in agreement with earlier treatments (as previously discussed by, e.g., M. Dixon & Webb, 1964, p. 134).

A previous attempt (Waley, 1953; see also M. Dixon & Webb, 1964, p. 140, eqn. IV. 178) to derive the steady-state rate equation for the kinetic model of Scheme I provided, instead of eqn. (7) or eqn. (16), an equation that predicts this simple dependence of $k_{\text{cat.}}/K_m$ only on B. This is because Waley (1953) made assumptions that effectively limited his steady-state analysis to the situation considered by Peller & Alberty (1959).

(III) The steady-state situation in which free reactant-state ionizations are not reflected in the pH-dependence of $k_{\text{cat.}}/K_m$. This situation requires terms B and D of eqn. (16) to be identical. This is a necessary but insufficient condition (see below). If $B \equiv D$, the rate constants for the direct formation of all three intermediate complexes (ES, EHS and EH_2S) must be identical, i.e. eqn. (21) must apply.

$$k_{+1} = k_{+2} = k_{+3} \quad (21)$$

There is no reason to suppose that the condition imposed by eqn. (21) must necessarily apply in the general case. The rate constants of eqn. (21) may characterize adsorptive processes, covalency changes or combinations of the two. Even if they characterize only adsorptive processes, the possibility that these may be of a multistep nature and the further possibility of pH-dependent conformational changes make the condition of eqn. (21) at least open to doubt.

In situations in which eqn. (21) does apply, however, eqn. (16) collapses to eqn. (22). Because of the interrelationships expressed in eqns. (17) and (18) and the condition imposed by eqn. (21), eqn. (22) may be written alternatively as eqn. (23):

$$\begin{aligned} k_{\text{cat.}}/K_m &= \frac{\tilde{k}_{\text{cat.}}/\tilde{K}_m}{1 + \frac{[\text{H}]}{K_{\text{esI}}} \cdot \left(\frac{k_{-1}}{k_{-2} + \tilde{k}_{\text{cat.}}} \right) + \frac{K_{\text{esII}}}{[\text{H}]} \cdot \left(\frac{k_{-3}}{k_{-2} + \tilde{k}_{\text{cat.}}} \right)} \quad (22) \end{aligned}$$

$$\begin{aligned} k_{\text{cat.}}/K_m &= \frac{\tilde{k}_{\text{cat.}}/\tilde{K}_m}{1 + \frac{[\text{H}]}{K_{\text{el}}} \cdot \left(\frac{k_{-2}}{k_{-2} + \tilde{k}_{\text{cat.}}} \right) + \frac{K_{\text{eil}}}{[\text{H}]} \cdot \left(\frac{k_{-2}}{k_{-2} + \tilde{k}_{\text{cat.}}} \right)} \quad (23) \end{aligned}$$

Eqns. (22) and (23) predict symmetrical bell-shaped profiles of $k_{\text{cat.}}/K_m$ against pH, which provide apparent acid dissociation constants. These may be regarded either as K_{esI} and K_{esII} , each associated with assemblies of rate constants (see eqn. 22), or as K_{el} and K_{eil} , each associated with different assemblies of rate constants (see eqn. 23).

Consideration of eqn. (22) reveals the situation in which a profile of $k_{\text{cat.}}/K_m$ against pH will provide values of K_{esI} and K_{esII} devoid of extraneous rate constants. The condition given by eqn. (24) is required in addition to that given by eqn. (22).

$$k_{-1} = k_{-3} = (k_{-2} + \tilde{k}_{\text{cat.}}) \quad (24)$$

These values of K_{esI} and K_{esII} , however, will be equal to those of K_{el} and K_{eil} if $\tilde{k}_{\text{cat.}} \ll k_{-2}$, i.e. if equilibrium obtains (see eqn. 23).

(c) *The forms of pH-dependence of $k_{\text{cat.}}/K_m$.* (I) The individual factors and their interaction. The pH-dependence of $k_{\text{cat.}}/K_m$ is contained in the term D/B·C of eqn. (16). As shown above, each of the factors B–D are of the form represented by X (eqn. 25) in which K_1 and K_2 are constants. Eqn. (16) therefore may be written as eqn. (26). In B, K_1 and K_2 [i.e. $K_{1\text{B}}$ and $K_{2\text{B}}$ of eqn. (26)] are free-reactant-state molecular (macroscopic) acid dissociation constants (K_{el} and K_{eil}), and in C and D they are apparent molecular constants containing rate constants additional to the various acid dissociation constants (K_{el} , K_{eil} , K_{esI} and K_{esII}).

Thus $K_{1\text{D}}$ can be expressed as

$$\frac{k_{+2}}{k_{+1}} \cdot K_{\text{el}} \quad \text{or as} \quad \frac{k_{-2}}{k_{-1}} \cdot K_{\text{esI}}$$

and $K_{1\text{C}}$ can be expressed as

$$\left(\frac{\tilde{k}_{\text{cat.}} + k_{-2}}{k_{-2}} \right) \cdot \frac{k_{+2}}{k_{+1}} \cdot K_{\text{el}}$$

or as

$$\left(\frac{\tilde{k}_{\text{cat.}} + k_{-2}}{k_{-1}} \right) \cdot K_{\text{esI}}$$

$$X = 1 + \frac{[\text{H}]}{K_1} + \frac{K_2}{[\text{H}]} \quad (25)$$

$$\frac{k_{\text{cat.}}}{K_m} = \frac{\frac{\tilde{k}_{\text{cat.}}}{\tilde{K}_m} \left(1 + \frac{[\text{H}]}{K_{1\text{D}}} + \frac{K_{2\text{D}}}{[\text{H}]} \right)}{\left(1 + \frac{[\text{H}]}{K_{1\text{B}}} + \frac{K_{2\text{B}}}{[\text{H}]} \right) \left(1 + \frac{[\text{H}]}{K_{1\text{C}}} + \frac{K_{2\text{C}}}{[\text{H}]} \right)} \quad (26)$$

The reciprocal of X represents the concentration of the intermediate protonic state of a dibasic acid, and its pH-dependence has the well-known bell-shaped form pointed out by Michaelis (1914). The pH-dependence of X (i.e. any one individual component of the D/B·C assembly) may be expressed (Haldane, 1930; H. Dixon, 1974) as $X = 1 + a \cosh x$,

Fig. 1. Forms of pH-dependence of $\log X$, where $X = 1 + ([H]/K_1) + (K_2/[H])$

The reciprocal of X represents the concentration of the intermediate (singly protonated) form of an acid of pK_a values pK_1 and pK_2 . (a) If $K_1 \gg K_2$ the normal pH-dependence is seen; there are three straight-line regions, of slopes -1 , 0 and $+1$, with the horizontal one at $\log X = 0$. The straight-line portions intersect at the pK_a values (M. Dixon, 1953). (b) If $K_2 > K_1$, which occurs with positively co-operative proton binding (H. Dixon, 1973, 1974), the straight-line portions no longer intersect at the pK_a values. The curve in (a) can be obtained by addition of two curves, each of which represents a single ionization, but the curve in (b) cannot; this is because X has real factors only if $K_1 \geq 4K_2$ (H. Dixon, 1975).

where $a = 2\sqrt{K_2/K_1}$ and $x = (\ln 10)(pH + \frac{1}{2} \log K_2 K_1)$, i.e. x is 2.303 times the difference between the pH and the mean of the two pK_a values.

To study the interaction of the factors B, C and D in eqn. (16) it is convenient to use logarithmic plots and rules for the changes in their slopes like those enunciated by M. Dixon (1953). The use of such plots is particularly valuable when factors are to be multiplied and divided as in $D/B \cdot C$, because the individual $\log X$ -pH plots can readily be added and subtracted by eye.

The plot of $\log X$ against pH shows, in general, three regions (Fig. 1a); at low pH it has a slope of -1 , and this slope changes twice as the pH is raised, to zero as pK_1 is passed and then to $+1$ as pK_2 is passed. These changes are not sharp, but the straight-line portions of the curve intersect if produced at pK_1 and at pK_2 (M. Dixon, 1953). It is also possible for pK_1

Fig. 2. One form of pH-dependence of $\log k_{cat.}/K_m$

$\log D/B \cdot C$ is the pH-dependent term of $\log k_{cat.}/K_m$ (eqn. 16). Since B, C and D all have the pH-dependences shown in Fig. 1, their logarithms may be added as shown here. For simplicity, straight lines have been drawn in the plots of the logarithms of the factors.

to exceed pK_2 ; this happens when $1/X$ represents the concentration of the intermediate form of a dibasic acid if the loss of the two protons is positively co-operative, so that the second dissociates more readily than the first. Such a situation changes the plot to that shown in Fig. 1(b); the lines no longer intersect at the pK_a values, but at their mean, where there is a 2-unit change of slope. In the subsequent discussion we shall assume, as is usually true, that pK_1 is below pK_2 . When this is not true, some of our statements about pK_a values will apply to the points of intersections of the lines rather than to the molecular pK_a values themselves.

Fig. 2 illustrates how the slopes of the plot of $\log D/B \cdot C$ against pH are derived from the slopes of the logarithms of the separate factors. At low pH the slope is $+1$, because both $-\log B$ and $-\log C$ have slopes of $+1$, and the slope of $\log D$ is -1 . As the pH is raised the slope changes six times, four times by -1 as the pK_a values in B and C are passed, and twice by $+1$ as those in D are passed, so that the final slope is -1 .

It is only pK_a values in B and C, not those in D, that are expressed as pK_a values in $D/B \cdot C$, in the sense of being 'mid-points' of sigmoid waves in the plot of $k_{cat.}/K_m$ against pH. These values will of course move up the curves away from the mid-points in the usual way as the pK_a assemblies characterizing rising and falling limbs of a profile move closer together (see below).

In Fig. 3, two pK_a values in B or C are shown four units apart. As each of them is passed there is a change of -1 in the slope of the plot of $\log D/B \cdot C$ against pH. If there were no intervening pK_a in D, they would be the pK_a values characterizing the two sides

Fig. 3. Effects on $k_{cat.}/K_m$ of pK_a values in function D

Two pK_a values in functions B and C have been arbitrarily set at pH 3 and pH 7. A pK_a in D has been set (a) at 6.0 and (b) at 6.5. Both the pK_a values in B and C (changes of -1 in the slope of the logarithmic plot) are expressed as mid-points of changes in $D/B \cdot C$; pK_a values in D determine where the steps concerned start, and hence the amplitude with which they are expressed.

of a bell-shaped curve. The pK_a value in D leads to a change of +1 in the slope. Its position determines the position of the start in the rise of the right-hand sigmoid curve, whereas the pK_a in B or C determines the position of the mid-point of this curve. Hence, although only pK_a values in B or C provide pK_a values in $D/B \cdot C$, those in D determine the extent to which the former are expressed, i.e. the height of the waves.

(II) The restriction on profile shape. It has been pointed out above that $C \equiv D$ if $\bar{k}_{cat.} \ll k_{-2}$. As $\bar{k}_{cat.}$ increases with respect to k_{-2} , so the pK_a values of C move further apart from those of D, symmetrically, since both move by $\log[(\bar{k}_{cat.} + k_{-2})/k_{-2}]$ (see eqns. 10 and 19). Since, therefore, pK_{1C} is this amount below pK_{1D} , and pK_{2C} is the same amount above pK_{2D} , the plots of $\log D$ and $-\log C$ against pH have a common centre about which they are symmetrical. This imposes a restriction on the possible shapes of pH-dependence that can occur. The general form can

be expressed as $D/B \cdot C = (1 + d \cosh x)/(1 + b \cosh(x + \beta))$, where x is 2.303 times the difference of the pH from the mean of the two pK_a values in C (or D), and β is a measure of the shift of the mean of the pK_a values in B from the mean of those in C and D. This expression shows that only four parameters are required to define the general shape of a profile of $k_{cat.}/K_m$ against pH. Two more are required to define a particular profile; one is the amplitude parameter, $\bar{k}_{cat.}/\bar{K}_m$, and the other is required to locate the profile on the pH axis, i.e. with respect to a pH of zero rather than with respect to the pH at which factors C and D are minimal (the arithmetic mean of pK_{1C} and pK_{2C} or of pK_{1D} and pK_{2D}).

Inspection of eqn. (16) might suggest that seven parameters are required to characterize a $(k_{cat.}/K_m)$ -pH profile, i.e. two apparent pK_a values in each of the factors B-D and $\bar{k}_{cat.}/\bar{K}_m$. That only six are required is a consequence of the common centre of symmetry of the plots of $\log D$ and $-\log C$ against pH.

(III) The permitted profile shapes. At equilibrium, as already stated, $k_{cat.}/K_m$ does not depend on pK_a values in C and D, and it is therefore independent of the relative magnitudes of k_{+1} , k_{+2} and k_{+3} . But as $\log[(k_{-2} + \bar{k}_{cat.})/k_{-2}]$ rises from zero, i.e. as the pK_a values in C and D draw apart, the relative rates of combination of S with E, EH and EH_2 become more important. The changes in slope of the plot of $\log D/B \cdot C$ against pH that are due to pK_a values in C and D must come in the sequence -1, +1, +1, -1, with equal intervals of $\log[(k_{-2} + \bar{k}_{cat.})/k_{-2}]$ between the first two and between the last two. There are therefore four possible arrangements in the order of slope changes in the complete function $\log D/B \cdot C$, according to the relative positions of pK_a values in B and those in C and D: (1) -1, -1, -1, +1, +1, -1, and its reverse; (2) -1, -1, +1, +1, -1, -1, and its reverse; (3) -1, +1, -1, -1, +1, -1; and (4) -1, -1, +1, +1, -1, -1. The possible shapes of curves that can be generated from these will be considered.

Arrangement (1) is that shown in Fig. 2. It gives a single unsymmetrical peak, with a plateau on one side, which like any of the waves or plateaus predicted, may be too low to be detectable. Arrangement (2) leads to a single sigmoid fall from the optimum on one side, and the possibility of three sigmoid steps on the other (Fig. 4). One of these arrangements is obtained when $k_{+3} < k_{+2} < k_{+1}$, i.e. when EH_2 combines faster than EH with S, and EH faster than E. Arrangement (3) can lead to a doubly sigmoid rise to the optimum on both sides; it is obtained when k_{+2} is larger than k_{+1} or k_{+3} . Arrangement (4) can lead to the more interesting shape of a double optimum, but this arrangement only arises in the unlikely circumstances that the pK_a values of D are closer together than those of B. Comparison of eqns. (9) and (11) shows that this means that k_{+3} and k_{+1} are

Fig. 4. A three-step pH-dependence of $k_{cat.}/K_m$

Changes of -1 in the slope of the log plot are labelled on the direct plot where they represent mid-points of sigmoid changes. Changes of $+1$ are labelled on the log plot and control the amplitudes of the sigmoid changes. The horizontal arrows on the log plot represent the magnitude of $\log(k_{cat.} + k_{-2})/k_{-2}$, by which the pK_a values of C are displaced outwards from those of D.

both larger than k_{+2} , which is the reason for the double optimum.

Now that the predictions have been made, the ways in which these are affected by deficiencies in the model can be considered. The model allows for the fact that the protonation and deprotonation that render EHS inert may not prevent (at least not completely) EH from combining with S. Nevertheless, there may be a further deprotonation of E or a further protonation of EH_2 that does render it incapable of binding S. The effect of this would be a further turn-down at the edges of the plots (a turn-down that might, of course, set in before one of those discussed). A further point is that there is not room in the range of pH stability of many enzymes for so many features as indicated in Fig. 4 to be clearly separated from one another.

(d) *The characteristics of profiles of $k_{cat.}/K_m$ against pH.* Features of rate-pH profiles of interest in addition to general shape are (1) the apparent pK_a values, (2) the pH-independent rate constants that charac-

terize the various plateau regions and (3) the width at half height of any clearly defined bell-shaped component of the profile.

The following discussion provides examples of the various situations that could obtain. The many other situations are readily analysed in a similar way by considering in turn certain portions of the profile assumed to be well separated from its other ionizations.

(I) Apparent pK_a values. Consider a profile like that given in Fig. 4 with slope changes in the order $-1, -1, +1, -1, +1, -1$ and assume that the rate optimum at the low-pH end of the profile is well separated from the other sigmoid waves in higher-pH regions. Because the optimum occurs in a pH region such that $[H] \gg K_{1D}, K_{2D}, K_{2B}, K_{2C}$, eqn. (26) provides the approximation given by eqn. (27), which can be rearranged to provide eqn. (28).

$$\frac{k_{cat.}}{K_m} = \frac{\frac{\tilde{k}_{cat.}}{\tilde{K}_m}}{\left(\frac{K_{1D}}{[H]}\right) \left(1 + \frac{[H]}{K_{1B}}\right) \left(1 + \frac{[H]}{K_{1C}}\right)} \quad (27)$$

$$\frac{k_{cat.}}{K_m} = \frac{\frac{\tilde{k}_{cat.}}{\tilde{K}_m} \left[\frac{K_{1B} \cdot K_{1C}}{K_{1D}(K_{1B} + K_{1C})} \right]}{1 + \frac{[H]}{(K_{1B} + K_{1C})} + \frac{K_{1B} \cdot K_{1C}/(K_{1B} + K_{1C})}{[H]}} \quad (28)$$

Eqn. (28) is the well-known form that describes a bell-shaped profile.

The apparent pK_a values are assemblies of K_{1B} and K_{1C} analogous to those that relate molecular pK_a values of a dibasic acid to the constituent group pK_a values (see Edsall & Wyman, 1958). As pointed out above, only K_{1B} and K_{1C} contribute to the expressions for the apparent pK_a values; K_{1D} appears only in the amplitude parameter based on $\tilde{k}_{cat.}/\tilde{K}_m$.

When, in addition, K_{1B} and K_{1C} are well separated, the expressions for the apparent K_a values in eqn. (28) reduce to K_{1B} and K_{1C} or K_{1C} and K_{1B} respectively (as shown in Fig. 4). If the special situation in which $k_{+2} = k_{+1}$ obtains, K_{1C} can be written as $K_{et}(k_{-2} + \tilde{k}_{cat.})/k_{-2}$ (see eqn. 23) and thus $K_{1C} > K_{1B}$ ($=K_{et}$). In this situation, the lower pK_a value of the bell has to be pK_{1C} .

When the order of slope changes is the one that provides a central bell with an additional sigmoid wave on each side $(-1, +1, -1, -1, +1, -1)$, the apparent pK_a values of the central bell are pK_{1B} and pK_{2B} , i.e. free-reactant-state pK_a values. Such pK_a values may be provided also by the arrangement that gives a profile like that in Fig. 2 if $pK_{1B} < pK_{2B} \ll pK_{1C}$. In the other types of profile, i.e. that in Fig. 4 and the double bell, the main bell-shaped components are described by K_{1B} and K_{1C} , and thus could arise from the ionization of the same group in the enzyme and in the enzyme-substrate complex. When a bell-shaped

profile represents a reaction occurring without change in the rate-limiting step over the pH range studied, this is generally considered to provide evidence that the reaction requires at least two groups, one in the acid form and one in the base form whose pK_a values lie somewhere between the pH values at the half-heights of the bell. The above treatment demonstrates that in the general steady-state situation this interpretation must be viewed with caution.

(II) pH-independent rate constants and change of rate-limiting step with pH. When all the pK_a values involved in a profile such as Fig. 4 are well separated, then plateau regions are formed, and values of $k_{cat.}/K_m$ can be found for each. The separation of the pK_a values of C from those of D means that $\tilde{k}_{cat.} \gg k_{-2}$ (see eqns. 10 and 19), and hence (eqn. 12) $\tilde{k}_{cat.}/\tilde{K}_m$ approaches k_{+2} .

If the order of pK_a values is that of Fig. 4, then the values of $D/B \cdot C$ for the three plateaus (from low to high pH) are K_{1B}/K_{1D} , unity and K_{2D}/K_{2B} . By using relationships like those given after the start of section 3(c)I, it can be shown that the values of $k_{cat.}/K_m$ in the three plateau regions become k_{+1} , k_{+2} and k_{+3} respectively. Clearly the plateaus are separated by pK_{1B} and pK_{2B} , and they represent the regions where the three forms EH_2 , EH and E predominate in turn. Over the whole pH range the binding of S is thus the rate-limiting step.

If, however, $pK_{1B} < pK_{1C}$, then on the highest plateau, i.e. the plateau at low pH, $D/B \cdot C$ is K_{1C}/K_{1D} , and the values for the other plateaus remain at unity and at K_{2D}/K_{2B} . Hence the value of $k_{cat.}/K_m$ on the highest plateau is $\tilde{k}_{cat.} k_{+2}/k_{-2}$. Of these terms, k_{+2}/k_{-2} represents the equilibrium constant for the formation of EHS from EH , the predominant form of free enzyme over the range of pH that represents this plateau as well as the central one (since the pH is above pK_{1B}). Thus the first step, the formation of EHS, has come to equilibrium, even though $\tilde{k}_{cat.} \gg k_{-2}$, and the second, the decomposition of EHS, has become rate-limiting. Lowering the pH to pass from the central plateau (above pK_{1C} and pK_{1D}) to the highest plateau accelerates the first step and its reverse by providing a faster pathway via EH_2 and EH_2S .

It can be shown that the formation of EHS from reactants may come into equilibrium even if $\tilde{k}_{cat.} \gg k_{-2}$ by using eqns. (10) and (19) to obtain eqn. (29).

$$\frac{D}{C} = \frac{k_{-2} + k_{-1} \frac{[H]}{K_{es1}} + k_{-3} \frac{K_{es11}}{[H]}}{\tilde{k}_{cat.} + k_{-2} + k_{-1} \frac{[H]}{K_{es1}} + k_{-3} \frac{K_{es11}}{[H]}} \quad (29)$$

The first-order rate constants $k_{-1}[H]/K_{es1}$, k_{-2} and $k_{-3}K_{es11}/[H]$, when multiplied by $[EHS]$ provide the rates of dissociation of S from EHS by the three routes via EH_2S , directly, and via ES , respectively.

Only one of these rate constants needs to be much larger than $\tilde{k}_{cat.}$ to make C and D identical, and thus to bring substrate binding into equilibrium, since from the steady-state equations

$$\frac{[EHS]}{[EH][S]_0} = \frac{k_{+2}D}{k_{-2}C}$$

Since, for the arrangement of ionizations just considered, $K_{1C} > K_{1D}$ and $K_{2D} < K_{2B}$, the relative magnitudes of the pH-independent rate constants are as required by the profile shown in Fig. 4. If instead, the order of the slope changes is the one that provides the profile with the double bell (i.e. $-1, -1, +1, +1, -1, -1$) then $K_{2D} > K_{2B}$, and this provides for the increase in the pH-independent rate constant on passing from the plateau region in the middle of the profile to the high pH optimum.

(III) Widths of bell-shaped profiles at half heights. One way that might be used in special cases to distinguish the situation in which a bell arises from two different free-reactant-state ionizations from that in which it could arise from the same group ionization in the free-reactant state and in the intermediate-complex state, is to examine the width of the profile at half height. When the two pK_a values are different molecular pK_a values of the free-reactant state, the width at half height can lie in the range 1.17–1.53 pH units if the protonic dissociations are subject to positive co-operativity (H. Dixon, 1973).

By contrast, when the two pK_a values arise one from term B and one from term C, they cannot give half heights closer than 1.53 units apart. H. Dixon (1975) pointed out that a term of the kind

$$\left(1 + \frac{[H]}{K_1} + \frac{K_2}{[H]}\right)$$

can be factorized into two terms $\left(1 + \frac{[H]}{P}\right)$ and $\left(1 + \frac{Q}{[H]}\right)$ that are real only if $K_1 \geq 4K_2$. Here the converse applies; multiplication of such factors by the process used in deriving eqn. (28) from eqn. (27) gives combined dissociation constants, which differ by at least a factor of 4. Thus one is $K_{1B} + K_{1C}$ and the other is $K_{1B} \cdot K_{1C}/(K_{1B} + K_{1C})$. Since K_{1B} is K_{e1} , and K_{1C} can be called $n \cdot K_{e1}$, the ratio between the combined constants is $(1+n)^2/n$, which has a minimum value of 4 when $n = 1$. In other words, curves like Fig. 1(b) cannot be obtained by adding together two curves that each express a single pK_a ; curves like Fig. 1(a) can.

(e) *A reappraisal of pH-rate profiles of enzyme reactions in the literature.* A non-equilibrium steady-state condition might be responsible for some of the many pH-rate profiles in the literature, determined at $[S]_0 < K_m$, that are not simple bell-shaped curves,

In the past, these have been attributed to other causes such as mixtures of enzymes, or specific buffer effects.

Examples of unsymmetrical pH-rate profiles are those reported by Brocklehurst *et al.* (1972), Barisas & McGuire (1974), McGregor *et al.* (1974) and Kwiatkowska *et al.* (1974).

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