The Effects of Hydrogen Ion Concentration on the Simplest Steady-State Enzyme Systems

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Laidler (1955) showed that consideration of the effect of pH on enzymic mechanisms that obey steady-state kinetics leads to the inclusion in the equations of a 'perturbation term' that can introduce curvature into the Lineweaver–Burk plots. He also stated conditions in which this term vanishes. This term can lead to apparent activation by substrate. Further, several cases are shown in which simplification, but not disappearance, of the perturbation term can lead to linearity of Lineweaver–Burk plots. These cases arise when the ionization of groups at the active site either is unaffected or is completely prevented when the enzyme–substrate complex is formed. It is also shown that $V_{(app.)}$ can vary with pH without a concomitant change in $K_{m(app.)}$ in certain cases that obey steady-state kinetics without implying that $K_m = K_s$. When the perturbation term is significant, Dixon's (1953) rules for the calculation of pK values will not always apply.

Enzymic reactions are affected by the H^+ ion concentration of the solvent. The theoretical description of the expected effects of the H^+ ion concentration on the two commonly used parameters of enzyme kinetics (the Michaelis constant and the maximum velocity) depends on the assumptions made. Such a series of assumptions might be the following.

(1) The enzyme reacts with a single substrate to give rise to products via intermediate enzyme–substrate complex(es).

(2) Enzyme is conserved (total enzyme concentration $[E]_t = [E]_{free} + \Sigma [ES \text{ complexes}]$).

(3) Substrate is present in a large excess over enzyme so that $[S]_{free} = [S]_{total}$ and so that a 'steady-state' is reached early in the course of the reaction.

(4) Only initial stages of the reaction are studied, so that those reactions involving products can be considered as being irreversible.

(5) No cofactors, inhibitors, activators or such effects ascribable to allosteric phenomena are present.

(6) The H^+ ion effects considered are such that, in their simplest form, two ionizing groups concerned with the enzymic activity are assumed to be present at the active site; that one (with dissociation constant K_1) has to be protonated and one (with dissociation constant K_2) has to be unprotonated for product formation to occur; that the substrate does not ionize and is bound in the enzyme–substrate complex by (an)other group(s) unaffected by pH [variations of assumption (6) are also considered].

(7) The rate of formation of products is so small that all forms of enzyme and enzyme–substrate

complexes are essentially at equilibrium with each other.

Such a system can be described diagrammatically as in Scheme 1. The reactions connecting substrata and ionization of the enzymically active site are depicted by arrows. The rate constants for these reactions are also shown (second-order ones with positive subscripts and first-order ones with negative subscripts). The first-order rate constant for the formation of products from the productive form of the enzyme–substrate complex is designated by k . Further, the following relations hold:

$$\begin{aligned} k_{-s}/k_{+s} &= K_s; & k_{-1}/k_{+1} &= K_1; & k_{-2}/k_{+2} &= K_2; \\ k'_{-s}/k'_{+s} &= K'_s; & k'_{-1}/k'_{+1} &= K'_1; & k'_{-2}/k'_{+2} &= K'_2; \\ k''_{-s}/k''_{+s} &= K''_s. \end{aligned}$$

Scheme 1. Reactions of the active site of an enzyme with protons and substrate. Abbreviations: E, enzyme; S, substrate; H, H^+ ion; EH, EH_2 , ES, EHS and EH_2S , various ionization forms of the enzyme and the enzyme–substrate complex.

As long as assumption (7) is made, the derivation of an equation relating the velocity of the reaction (i.e. the rate of product formation or of substrate disappearance, v) to the substrate concentration and H^+ ion concentration gives rise to no problems whatsoever. Such an equation can be found wherever enzyme kinetics are considered, and has the form shown in eqn. (1):

$$\frac{[E]_t}{v} = \frac{1}{k} \left(1 + \frac{K'_1}{[H^+]} + \frac{[H^+]}{K'_2} \right) + \frac{1}{k} \left(1 + \frac{K_1}{[H^+]} + \frac{[H^+]}{K_2} \right) \frac{K_s}{[S]} + \beta \quad (1)$$

where $\beta = 0$.

If, however, assumption (7) is not made, the derivation of steady-state equations for such a system as that shown in Scheme 1 leads to rather involved expressions for the reaction velocity (Laidler, 1955; Hearon, Bernhard, Friess, Botts & Morales, 1959). Such derivations can be carried out by the method of determinants or, much more easily, by using the schematic method of King & Altman (1956) (which is the method used in the present work).

The reactions leading directly from EH_2 to EH_2S and from E to ES are usually neglected, under the assumption that proton-transfer reactions are all much faster than the release of either substrate or product from the enzyme-substrate complex. This assumption cannot be generally valid. For example, a dissociating histidine residue on the enzyme might have a dissociation constant $K_a = k_{-a}/k_{+a} = 10^{-7}$ M. The protonation of such a group can occur with a second-order reaction rate constant (k_{+a}) of about $10^{10} \text{ s}^{-1} \cdot \text{M}^{-1}$ (Eigen & Hammes, 1963). This will make k_{-a} about 10^3 s^{-1} . Several examples of enzymes showing enzyme-substrate dissociation constants and/or rates of product formation of this same order of magnitude (or larger) are listed by Eigen & Hammes (1963). It is therefore necessary to take into account all the reaction pathways indicated in Scheme 1.

In the system of Scheme 1, and making assumptions (1)–(6), the steady-state expression relating reaction rate to substrate and H^+ ion concentrations is a very discouraging one: the rate of reaction is proportional to a fraction with a numerator containing the sum of 15 terms, each made up of five rate constants as well as terms for substrate concentration of up to the third power, as well as H^+ ion concentration terms of up to the fourth power; the denominator of the fraction contains 117 such terms. Although the expression can presumably be simplified to some extent by factorization, its use in practice would almost invariably be shunned. As shown by Laidler (1955), the expression obtained describing the reaction velocity in such a system

still obeys eqn. (1), but β now is not zero: it is a perturbation term containing higher powers of $[S]$ and $[H^+]$ in both the numerator and denominator.

There exist certain conditions under which the perturbation term will disappear, and Laidler (1955) has pointed out the only two plausible cases: (1) when $k \ll k_{-s}$, k'_{-s} , k''_{-s} ; i.e. all forms of the enzyme and the enzyme-substrate complex are in equilibrium (or quasi-equilibrium at least) with each other, and this equilibrium remains unaffected by the process of product formation; (2) the binding of substrate is competitive with respect to the ionizations ($K'_1 = 0$ and $K'_2 = \infty$).

The Briggs & Haldane (1925) modification of the Michaelis & Menten (1913) equation states that:

$$\frac{[E]_t}{v} = \frac{1}{k_{(app.)}} \left(1 + \frac{k_{-s(app.)} + k_{(app.)}}{k_{+s(app.)}[S]} \right) \quad (2)$$

where v is the experimentally determined rate of reaction at a specific pH, and $k_{(app.)}$, $k_{-s(app.)}$ and $k_{+s(app.)}$ are pH-dependent terms related to k , k_{-s} and k_{+s} of Scheme 1 and eqn. (1).

Experimentally it is common practice to treat initial rate data by plotting $1/v$ against $1/[S]$ (Lineweaver & Burk, 1934). Two parameters from such plots are used in the standard description of properties of enzymes: the slope $[(k_{-s(app.)} + k_{(app.)})/k_{+s(app.)}[E]_t k_{(app.)}]$ and the y -axis intercept ($=$ apparent maximum velocity under the conditions of the experiment $= V_{(app.)} = [E]_t k_{(app.)}$). The ratio of slope/intercept is operationally defined as the $K_{m(app.)}$ (the apparent Michaelis constant). If the perturbation term, β , makes a significant contribution to eqn. (1), it may make plots of $1/v$ against $1/[S]$ non-linear. Eqn. (2) will then obviously not apply to such a system.

There is, further, at least one set of conditions under which the perturbation term simplifies to manageable proportions even though it may not actually disappear. This occurs when the dissociation constants of groups at the active site either remain unaltered on formation of the enzyme-substrate complex or they are abolished. [A well-studied example of such a class of enzymes is α -chymotrypsin (Laidler & Barnard, 1956; Bender, Clement, Kézdy & Heck, 1964).] As the equations relevant to such cases have not been reported before, they are now described in some detail, especially since such steady-state systems exhibit rather curious, indeed almost bizarre, properties. An examination of the equations to be given will show what effects the simplified form of β will have on the slope and intercepts (and hence on the $V_{(app.)}$ and $K_{m(app.)}$) of Lineweaver-Burk plots under various conditions of pH and substrate concentration for which these plots remain linear.

(A) $K_1 = K'_1$ and $K_2 = K'_2$: binding of substrate has no effect on ionizations at the active centre

If to the system represented in Scheme 1 the restriction is imposed such that substraction of the active site will not affect the ionization processes of groups therein, it follows from the principles of microscopic reversibility that the ionization of such groups will not affect the substraction reaction. The following conditions will exist:

$$\begin{aligned} k_{+1} &= k'_{+1}; & k_{-1} &= k'_{-1}; & K_1 &= K'_1; \\ k_{+2} &= k'_{+2}; & k_{-2} &= k'_{-2}; & K_2 &= K'_2; \\ k_{+s} &= k'_{+s} = k''_{+s}; & k_{-s} &= k'_{-s} = k''_{-s}; & K_s &= K'_s = K''_s \end{aligned}$$

The equation relating the reaction rate, v , to substrate and H^+ ion concentrations as derived by

from the following considerations. β is obviously $\leq f(1/k_{+s}[S])$. When this is compared with $f(k_{-s}/k_{+s}[S]k)$ in eqn. (3) (where k_{-s}/k_{+s} has been substituted for K_s), it is obvious that, as long as $k \ll k_{-s}$, β will perforce be negligible compared with the other terms in eqn. (3), and this holds true for all values of $[S]$ and $[H^+]$.

(2) When $[H^+] \gg K_1$ and K_2 : pH low compared with pK values of groups. Here: $k_{+1}[H^+] \gg k_{-1}$ and $k_{+2}[H^+] \gg k_{-2}$. It follows also that:

$$k_{+1}[H^+]k_{+2}[H^+] \gg k_{-1}k_{-2} \text{ and } k_{+1}[H^+]k_{-2}$$

Under these conditions β can be simplified, as certain terms in it will now lose their significance relative to the rest of the expression. Eqn. (4) is thus reduced to:

$$\beta = \frac{k_{+1}[H^+]k_{+2}[H^+](k_{+s}[S] + k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}{k_{+s}[S]k_{+1}[H^+]k_{-2}(k_{+s}[S] + k_{-s} + k_{+2}[H^+])(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}$$

so that:

$$\beta = \frac{k_{+2}[H^+]}{k_{-2}} \cdot \frac{1}{k_{+s}[S]} \cdot \frac{(k_{+s}[S] + k_{-s} + k_{-2})}{(k_{+s}[S] + k_{-s} + k_{+2}[H^+])}$$

the method of King & Altman (1956) reduces to the following:

$$\frac{[E]_t}{v} = f \left(1 + \frac{K_s}{[S]} \right) \frac{1}{k} + \beta \quad (3)$$

where

$$f = 1 + \frac{K_1}{[H^+]} + \frac{[H^+]}{K_2}$$

The perturbation term, β , has the following form:

This can be split up into the sum of two fractions (making also use of the equalities $k_{-s}/k_{+s} = K_s$ and $k_{-2}/k_{+2} = K_2$):

$$\beta = \left(1 + \frac{K_s}{[S]} \right) \frac{[H^+]}{K_2} \cdot \frac{1}{(k_{+s}[S] + k_{-s} + k_{+2}[H^+])} \quad (a)$$

$$+ \frac{1}{k_{+s}[S]} \cdot \frac{k_{+2}[H^+]}{(k_{+s}[S] + k_{-s} + k_{+2}[H^+])} \quad (b)$$

$$\beta = \frac{(k_{-1}k_{-2} + k_{+1}[H^+]k_{-2} + k_{+1}[H^+]k_{+2}[H^+])(k_{+s}[S] + k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}{k_{+1}[H^+]k_{-2}k_{+s}[S]\{(k_{+s}[S] + k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+]) + (k_{+s}[S] + k_{-s} + k_{-2})k_{-1} + (k_{+s}[S] + k_{-s} + k_{+1}[H^+])k_{+2}[H^+]\}}$$

which is the same as:

$$\beta = f \frac{1}{k_{+s}[S]} \cdot \frac{(k_{+s}[S] + k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}{\{(k_{+s}[S] + k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+]) + (k_{+s}[S] + k_{-s} + k_{-2})k_{-1} + (k_{+s}[S] + k_{-s} + k_{+1}[H^+])k_{+2}[H^+]\}} \quad (4)$$

At first sight it might still appear a hopeless task to try to obtain useful information from ordinary Lineweaver & Burk (1934) plots for such a system, since the presence of higher powers of $[S]$ in both the numerator and denominator of β indicates that straight lines will not be obtained in plots of $1/v$ against $1/[S]$. This, however, is not necessarily the case, and there are conditions under which $1/v$ will be proportional to $1/[S]$. These conditions, for linear Lineweaver-Burk plots, are as follows.

(1) When $k \ll k_{-s}$: β becomes negligible. This is equivalent to the quasi-equilibrium condition. Lineweaver-Burk plots will be linear as can be seen

When these are compared with the main body of eqn. (3) certain simplifications become obvious. Fraction (a) becomes much smaller than:

$$(1/k)(1 + K_s/[S])([H^+]/K_2)$$

as soon as $k_{+2}[H^+]$ becomes large enough to make:

$$1/(k_{+s}[S] + k_{-s} + k_{+2}[H^+]) \ll 1/k$$

Irrespective of the relative sizes of k_{-s} and k , a H^+ ion concentration will be reached at which the above inequality becomes true [k_{+2} , like k_{+1} , has a value in the neighbourhood of $10^{10} \text{ s}^{-1} \cdot \text{M}^{-1}$ (Eigen

& Hammes, 1963), and k would be expected to be very much smaller than this].

Fraction (b) must be equal to or smaller than $1/k_{+s}[S]$ (depending on the relative size of $k_{+2}[H^+]$ and $k_{+s}[S] + k_{-s}$ in the denominator of the fraction). Fraction (b) will necessarily become negligible compared with $(K_s/[S])([H^+]/K_2)(1/k)$

$$[\text{i.e. } (k_{-s}/k_{+s}[S])([H^+]/K_2)(1/k)]$$

as soon as $(k_{-s}/k)([H^+]/K_2)$ becomes much larger than 1, and this will occur at low enough pH, even if $k \gg k_{-s}$.

The perturbation term in eqn. (3) can be expected to become negligible at 'very high' H^+ ion concentrations, the exact meaning of 'very high' depending on the relative values of k and k_{-s} . Under these conditions of pH, therefore, eqn. (3) becomes:

$$\frac{[E]_t}{v} = \frac{1}{k} \cdot \frac{[H^+]}{K_2} \left(\frac{K_s}{[S]} + 1 \right) \quad (5)$$

(3) When $K_2 \gg [H^+] \gg K_1$. Provided that K_2 and K_1 are very different, at H^+ ion concentrations intermediate between them, the perturbation term will simplify. Under such conditions eqn. (3) becomes:

$$\frac{[E]_t}{v} = \frac{1}{k} \left(1 + \frac{K_s}{[S]} \right) + \beta \quad (6)$$

As $k_{+1}[H^+] \gg k_{-1}$ and $k_{-2} \gg k_{+2}[H^+]$, some terms in eqn. (4) are insignificant with respect to the rest of the expression and the whole will simplify to:

$$\beta = \frac{k_{+1}[H^+] k_{-2}(k_{+s}[S] + k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}{k_{+s}[S] k_{+1}[H^+] k_{-2}(k_{+s}[S] + k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}$$

or

$$\frac{1}{k_{+s}[S]} \cdot \frac{k_{-1}}{k_{+s}[S] + k_{-s} + k_{-1}}$$

$$\frac{1}{k_{+s}[S] + k_{-s} + k_{-1}}$$

where $K_m = (k_{-s} + k)/k_{+s}$, which is the definition of the Michaelis constant as given by Briggs & Haldane (1925).

When the H^+ ion concentration is very low, β will not be amenable to any useful simplification unless some added restrictions are introduced into the system.

(4) When $[H^+] \ll K_1$ and K_2 and $(k_{-s} + k_{-1}) \gg k$. Eqn. (3) now becomes:

$$\frac{[E]_t}{v} = \frac{1}{k} \cdot \frac{K_1}{[H^+]} \left(1 + \frac{K_s}{[S]} \right) + \beta \quad (8)$$

Because of the limiting conditions of H^+ ion concentration, $k_{-1} \gg k_{+1}[H^+]$ and $k_{-2} \gg k_{+2}[H^+]$, so that β reduces to:

$$\beta = \frac{k_{-1}}{k_{+1}[H^+]} \cdot \frac{1}{k_{+s}[S]} \cdot \frac{(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}{(k_{+s}[S] + k_{-s} + k_{-1})}$$

This can be split up into the sum of two fractions, thus:

$$\beta = \left(1 + \frac{K_s}{[S]} \right) \frac{K_1}{[H^+]} \cdot \frac{1}{k_{+s}[S] + k_{-s} + k_{-1}} \quad (c)$$

$$+ \frac{1}{k_{+s}[S]} \cdot \frac{k_{-1}}{k_{+s}[S] + k_{-s} + k_{-1}} \quad (d)$$

When the further limitation is introduced that $(k_{-s} + k_{-1}) \gg k$, fraction (c) obviously becomes insignificant compared with $(1/k)(K_1/[H^+])(1 + K_s/[S])$ from the main body of eqn. (8). Fraction (d) can be compared with $(K_1/[H^+])(K_s/[S])(1/k)$ from eqn. (8), i.e.:

$$\text{versus } \frac{k_{-s}}{k_{+s}[S]} \cdot \frac{k_{-1}}{k_{+1}[H^+] k}$$

$$\text{versus } \frac{k_{-s}}{k_{+1}[H^+] k}$$

which equals $1/k_{+s}[S]$. Under these conditions of H^+ ion concentration, therefore, eqn. (6) becomes:

$$\frac{[E]_t}{v} = \frac{1}{k} \left(\frac{K_s}{[S]} + 1 \right) + \frac{1}{k_{+s}[S]}$$

and as $K_s = k_{-s}/k_{+s}$:

$$\frac{[E]_t}{v} = \frac{k_{-s}}{k_{+s}[S]} \cdot \frac{1}{k} + \frac{k}{k_{+s}[S] k} + \frac{1}{k}$$

which can be written as:

$$\frac{[E]_t}{v} = \frac{1}{k} \left(1 + \frac{K_m}{[S]} \right) \quad (7)$$

Provided that $[H^+]$ becomes small enough, $k_{-s}/k_{+1}[H^+] k$ must become much larger than $1/(k_{+s}[S] + k_{-s} + k_{-1})$, thus allowing the whole of the perturbation term to become negligible with respect to the rest of eqn. (8). Eqn. (9) will thus represent the reaction under these limiting conditions:

$$\frac{[E]_t}{v} = \frac{1}{k} \cdot \frac{K_1}{[H^+]} \left(1 + \frac{K_s}{[S]} \right) \quad (9)$$

There are at least two sets of restrictive conditions that will allow β to cease to function as a perturbation term at all H^+ ion concentrations and for all

values of k/k_{-s} for which this ratio is not much less than 1. These conditions are as follows.

(5) When $(k_{-1} + k_{-s}) \gg k_{+s}[S]$ and $(k_{-2} + k_{-s}) \gg k_{+s}[S]$. This allows the expression for β of eqn. (3) to be reduced to:

$$\beta = f \frac{1}{k_{+s}[S]} \cdot \frac{(k_{-s} + k_{-2})(k_{+s}[S] + k_{-s} + k_{+1}[H^+])}{(k_{-s} + k_{-2})(k_{-s} + k_{+1}[H^+] + k_{-1}) + (k_{-s} + k_{+1}[H^+] + k_{+s}[S])k_{+2}[H^+]}$$

The denominator of β contains $k_{+s}[S]k_{+2}[H^+]$ as a term, and it is of interest to determine whether this term can be considered to be insignificant with respect to the rest of the denominator.

(i) When $[H^+] < K_2$ (so that $k_{-2} \gg k_{+2}[H^+]$): under these conditions $k_{+s}[S]k_{+2}[H^+] \ll k_{-2}(k_{+s} + k_{-1})$, since by definition $(k_{-s} + k_{-1}) \gg k_{+s}[S]$.

(ii) When $[H^+] > K_2$ (so that $k_{+2}[H^+] > k_{-2}$): the lack of significance of $k_{+s}[S]k_{+2}[H^+]$ is uncertain. If k_{+1} and k_{+2} are nearly equal (which is not unlikely; Eigen & Hammes, 1963), so that $k_{+1}[H^+]$ is now also $\gg k_{-1}$, or $\gg k_{-2}$, then:

$$k_{+s}[S]k_{+2}[H^+] \ll (k_{-s} + k_{+1}[H^+])k_{+2}[H^+]$$

It is also possible that in this region of H^+ ion concentration $k_{+s}[S]k_{+2}[H^+] \ll (k_{-s} + k_{-2})k_{+1}[H^+]$. If any of these inequalities is satisfied, $k_{+s}[S]k_{+2}[H^+]$ will be insignificant with respect to the rest of the denominator of β also when $[H^+] > K_2$.

(iii) When $[H^+] \gg K_2$ (and also much larger than K_1), $[E]_t/v$ becomes independent of β , as was shown in eqn. (5).

Thus, except possibly for that region of H^+ ion concentration where $[H^+]$ is larger, but not much larger, than K_2 , $k_{+s}[S]k_{+2}[H^+]$ can be neglected as a term in the denominator of β whenever $k_{-s} + k_{-1}$ and $k_{-s} + k_{-2}$ are much larger than $k_{+s}[S]$. The perturbation term can then be written as the sum of two fractions:

$$\beta = f \frac{(k_{-s} + k_{-2})}{(k_{-s} + k_{-2})(k_{-s} + k_{+1}[H^+] + k_{-1}) + (k_{-s} + k_{+1}[H^+])k_{+2}[H^+]} + f \frac{1}{k_{+s}[S]} \cdot \frac{(k_{-s} + k_{-2})(k_{-s} + k_{+1}[H^+])}{(k_{-s} + k_{-2})(k_{-s} + k_{+1}[H^+] + k_{-1}) + (k_{-s} + k_{+1}[H^+])k_{+2}[H^+]}$$

It is now obvious that under these conditions β will not introduce any curvature in plots of $1/v$ against $1/[S]$, since the first fraction is independent of $[S]$ and the second one is proportional to $1/[S]$. The two fractions will, however, contribute to the y-axis intercept and to the slope respectively of such a plot.

(6) When $(k_{-s} + k_{-2}) \gg k_{+s}[S]$ and $(k_{+s}[S] + k_{-s}) \gg k_{-1}$

(i.e. K_1 and K_2 are far apart). β will now simplify to:

$$f \frac{1}{k_{+s}[S]} \cdot \frac{k_{-s} + k_{-2}}{k_{-s} + k_{-2} + k_{+2}[H^+]} \quad (11)$$

and as such will not produce any curvature in plots of $1/v$ against $1/[S]$.

(B) $K'_1 = 0$ and $K_2 = K'_2$: binding of substrate restricts an ionization at the active centre

If in the active site of the free enzyme that group with dissociation constant K_1 reacts, in its protonated form, with a molecule of substrate to form the enzyme-substrate complex, the dissociation of such a group will be abolished (as suggested, for example, for chymotrypsin by Wedler & Bender, 1969). Such a system is represented by Scheme 2 and the equation describing the rate of reaction for such a system is:

$$\frac{[E]_t}{v} = \left(1 + \frac{[H^+]}{K_2} + f \frac{K_s}{[S]}\right) \frac{1}{k} + f \frac{1}{k_{+s}[S]} \cdot \frac{k_{+s}[S] + k_{-s} + k_{-2}}{k_{+s}[S] + k_{-s} + k_{-2} + k_{+2}[H^+]} \quad (12)$$

In this case plots of $1/v$ against $1/[S]$ will be linear under the following conditions.

(1) When $[H^+] \gg K_1$ and K_2 : pH low compared with pK values of groups. Eqn. (12) reduces to:

$$\frac{[E]_t}{v} = \frac{1}{k} \cdot \frac{[H^+]}{K_2} \left(1 + \frac{K_s}{[S]}\right) \quad (5)$$

and β is negligible, as was true for the system in Scheme 1, and for identical reasons.

(2) When $K_2 \gg [H^+] \gg K_1$. Eqn. (12) reduces to:

$$\frac{[E]_t}{v} = \left(1 + \frac{K_m}{[S]}\right) \frac{1}{k} \quad (7)$$

(where K_m is the Michaelis constant as defined by

Scheme 2. Reactions of the active site with protons and substrate. Binding of substrate restricts the deprotonation of the group with the highest pK. Abbreviations as in Scheme 1.

Briggs & Haldane, 1925), as was true for the system in Scheme 1, and for the same reasons.

(3) When $[H^+] \ll K_1$ and K_2 . Eqn. (12) reduces to:

$$\frac{[E]_t}{v} = \left(1 + \frac{K_m}{[S]} \cdot \frac{K_1}{[H^+]}\right) \frac{1}{k} \quad (13)$$

irrespective of the relative values of k , k_{-1} and k_{-2} .

(4) When $(k_{-s} + k_{-2}) \gg k_{+s}[S]$. Eqn. (12) reduces to:

$$\frac{[E]_t}{v} = \left(1 + \frac{[H^+]}{K_2} + f \frac{K_s}{[S]}\right) \frac{1}{k} + f \frac{1}{k_{+s}[S]} \cdot \frac{k_{-s} + k_{-2}}{k_{-s} + k_{-2} + k_{+2}[H^+]} \quad (14)$$

so that plots of $1/v$ against $1/[S]$ will give straight lines at all H^+ ion concentrations.

(C) $K_1 = K'_1$ and $K'_2 = \infty$: binding of substrate restricts protonation at the active site

If in the active site of the free enzyme that group with dissociation constant K_2 reacts, in its unprotonated form, with a molecule of substrate to form the enzyme-substrate complex, the dissociation of such a group will be abolished, thus resulting in a system such as that shown in Scheme 3. The rate of reaction for such a system will be described by the following equation:

$$\frac{[E]_t}{v} = \left(1 + \frac{K_1}{[H^+]} + f \frac{K_s}{[S]}\right) \frac{1}{k} + f \frac{1}{k_{+s}[S]} \cdot \frac{k_{+s}[S] + k_{-s} + k_{+1}[H^+]}{k_{+s}[S] + k_{-s} + k_{+1}[H^+] + k_{-1}} \quad (15)$$

In this case straight lines will be obtained in plots of $1/v$ against $1/[S]$ under the following conditions.

(1) If $[H^+] \gg K_1$ and K_2 :

$$\frac{[E]_t}{v} = \left(1 + \frac{K_m}{[S]} \cdot \frac{[H^+]}{K_2}\right) \frac{1}{k} \quad (16)$$

Scheme 3. Reactions of the active site with protons and substrate. Binding of substrate restricts the protonation of the group with the lowest pK. Abbreviations as in Scheme 1.

(2) If $K_2 \gg [H^+] \gg K_1$:

$$\frac{[E]_t}{v} = \left(1 + \frac{K_m}{[S]}\right) \frac{1}{k} \quad (7)$$

In alkaline medium simplification of the expression for β depends on some further restriction being introduced into the system, as, for example, in the following case.

(3) K_2 and $K_1 \gg [H^+]$ and $(k_{-s} + k_{-1}) \gg k$. In this case, because of arguments used above [Case (A)(4)], β will disappear and:

$$\frac{[E]_t}{v} = \frac{1}{k} \cdot \frac{K_1}{[H^+]} \left(1 + \frac{K_s}{[S]}\right) \quad (9)$$

Further possibilities of simplification of β exist under such circumstances where straight lines in plots of $1/v$ against $1/[S]$ are obtained at all H^+ ion concentrations. These are as follows.

(4) $k_{-s} + k_{-1} \gg k_{+s}[S]$. In such a case:

$$\frac{[E]_t}{v} = \frac{1}{k} \left(1 + \frac{K_1}{[H^+]} + f \frac{K_s}{[S]}\right) + f \frac{1}{k_{-s} + k_{-1} + k_{+1}[H^+]} + f \frac{1}{k_{+s}[S]} \cdot \frac{k_{-s} + k_{+1}[H^+]}{k_{-s} + k_{-1} + k_{+1}[H^+]} \quad (17)$$

(5) $k_{-1} \ll (k_{-s} + k_{+s}[S])$. The perturbation term will now simplify to $f/k_{+s}[S]$, so that the equation relating the reaction velocity to substrate concentration and to H^+ ion concentration will be:

$$\frac{[E]_t}{v} = \frac{1}{k} \left(1 + \frac{K_1}{[H^+]} + f \frac{K_m}{[S]}\right) \quad (18)$$

(D) $K'_1 = 0$ and $K'_2 = \infty$: binding of substrate abolishes both ionizations at the active site

If the ionization of both ionizing groups in the active site is suppressed on formation of the enzyme-substrate complex, the reaction system will be

Scheme 4. Reactions of the active site with protons and substrate. Binding of substrate prevents the ionization of both groups. Abbreviations as in Scheme 1.

represented by Scheme 4, for which the steady-state equation is:

$$\frac{[\text{E}]_t}{v} = \left(1 + f \frac{K_m}{[\text{S}]} \right) \frac{1}{k} \quad (19)$$

In this case there is no perturbation term; the system corresponds to one of the two cases described by Laidler (1955) for which the perturbation term disappears at all values of H^+ ion concentration.

Consequences and conclusions

It must be stressed again that the foregoing discussion is limited to very simple systems with a serious restriction on the dissociation constants of ionizing groups at the enzymically active site, with but a single substrate, and with a single activated enzyme-substrate complex that gives rise to products without an intermediate isomerization to an enzyme-product complex. The equations that have been derived are nevertheless quite instructive and several interesting conclusions can be drawn from them.

(1) The definition of the Michaelis constant derived (from even simpler systems than those considered here) by Briggs & Haldane (1925), namely that $K_m = (k_{-s} + k)/k_{+s}$, is valid only under very special conditions. Even if $k_{-s} \ll k$, the Michaelis constant obtainable experimentally (e.g. from Lineweaver-Burk plots) can, under the appropriate conditions of pH [e.g. Case (A) (2) and (4); Case (B) (1); Case (C) (3)], be equal to the dissociation constant of the enzyme-substrate complex, K_s .

(2) Substrate activation effects. Fig. 1 shows a Lineweaver-Burk plot calculated from eqn. (3) when $\beta \neq 0$ by using the constants shown in the figure legend: it is typical of the results obtained with many enzyme systems.

Fig. 2 shows the variation of reaction velocity with a very wide range of substrate concentration calculated from eqn. (15), a case where substratum restricts protonation: this figure can be usefully compared with Fig. 2 (insert), which is redrawn from Trowbridge, Krehbiel & Laskowski (1963).

Fig. 1. Apparent substrate activation. The line was calculated by use of eqns. (3) and (4). $k_{-s} = 10^2 \text{ s}^{-1}$; $k_{+s} = 10^5 \text{ s}^{-1} \cdot \text{M}^{-1}$; $k = 10^4 \text{ s}^{-1}$; $k_{-1} = 10^3 \text{ s}^{-1}$; $k_{+1} = 10^{10} \text{ s}^{-1} \cdot \text{M}^{-1}$; $k_{-2} = 10^7 \text{ s}^{-1}$; $k_{+2} = 10^{10} \text{ s}^{-1} \cdot \text{M}^{-1}$; pH 9.0.

Fig. 2. Effect of substrate concentration on reaction velocity. The line was calculated by using eqn. (15). $k_{-s} = 1 \text{ s}^{-1}$; $k_{+s} = 10^5 \text{ s}^{-1} \cdot \text{M}^{-1}$; $k = 10^3 \text{ s}^{-1}$; $k_{-1} = 10^3 \text{ s}^{-1}$; $k_{+1} = 10^{10} \text{ s}^{-1} \cdot \text{M}^{-1}$; $K_2 > K_1$; pH 10.0. The insert is redrawn from Trowbridge *et al.* (1963).

Fig. 3. Effect of pH on (a) $pK_{m(app.)}$ (i.e. $-\log K_{m(app.)}$) and on (b) $\log V_{(app.)}$. The continuous lines (—) were calculated from plots of $1/v$ against $1/[S]$ obtained by using eqns. (3) and (4). The broken lines (---) are drawn according to the rules of Dixon (1953). $(k_{-1} + k_{-2})$ and $(k_{-2} + k_{-3}) \gg k_{+3}[S]$; $k_{-3} = 10^2 s^{-1}$; $k_{+3} = 10^5 s^{-1} \cdot M^{-1}$; $k = 10^3 s^{-1}$; $k_{-1} = 10^4 s^{-1}$; $k_{+1} = 10^{10} s^{-1} \cdot M^{-1}$; $k_{-2} = 10^7 s^{-1}$; $k_{+2} = 10^{10} s^{-1} \cdot M^{-1}$; substrate concentration varied between 1 mM and 10 mM (at $pH \geq 7$) and between 1 mM and 50 mM (at $pH < 7$).

Fig. 4. Effect of pH on (a) $pK_{m(app.)}$ (i.e. $-\log K_{m(app.)}$) and on (b) $\log V_{(app.)}$. The continuous lines (—) were calculated from plots of $1/v$ against $1/[S]$ obtained by using eqn. (18). The broken lines (---) are drawn according to the rules of Dixon (1953). $(k_{+3}[S] + k_{-3}) \gg k_{-1}$; $K_1 = K'_1 = 10^{-8} M$; $k_{-1} = 10^2 s^{-1}$; $k_{+1} = 10^{10} s^{-1} \cdot M^{-1}$; $K_2 = 10^{-4} M$; $K'_2 = \infty$; $k_{-2} = 10^4 s^{-1}$; $k_{+2} = 10^6 s^{-1} \cdot M^{-1}$; $k = 10^5 s^{-1}$; substrate concentration was 10 mM and above.

Table 1. Comparison of the values of rate and equilibrium constants used in the construction of the plots in Fig. 3 and the values calculated from these plots assuming quasi-equilibrium.

$(k_{-3} + k_{-1})$ and $(k_{-3} + k_{-2}) \gg k_{+3}[S]$.		
Constant	True value	Value assuming quasi-equilibrium
pK_1	7.00	6.95
pK'_1	7.00	5.95
pK_2	3.00	2.00
pK'_2	3.00	3.00
k_{-3}	$10^2 s^{-1}$	—
k_{+3}	$10^5 s^{-1} \cdot M^{-1}$	—
k	$10^3 s^{-1}$	$10^3 s^{-1}$
K_s	$10^{-3} M$	$1.1 \times 10^{-2} M$
K_m	$1.1 \times 10^{-2} M$	$1.1 \times 10^{-2} M$

(3) Effects of pH on the experimentally determined Michaelis constant ($K_{m(app.)}$) and maximum reaction velocity ($V_{(app.)}$). I have chosen two examples to illustrate cases that might arise in

practice and for which interpretations are possible according to either steady-state or quasi-equilibrium assumptions.

The results in Fig. 3 have been calculated with the use of eqns. (3) and (4) and by using the constants with values as given in the figure legend. The concentrations of substrate used for these calculations covered a tenfold concentration range, and were in the neighbourhood of the value of the $K_{m(app.)}$. It may be noted that within these limits Lineweaver-Burk plots were linear. Table 1 shows a comparison of true constants for the system (i.e. those used for the calculation of the results) and their values as estimated by Dixon's (1953) rules assuming quasi-equilibrium to be operative with the system. There is a considerable discrepancy between the two sets of results.

A second example is depicted in Fig. 4, and Table 2 compares the true values of the various constants with those obtained by assuming quasi-equilibrium to be operative. The discrepancies here are limited to the values obtained for the K_s , but other impli-

Table 2. Comparison of the values of the rate and equilibrium constants used in the construction of the plots in Fig. 4 and the values calculated from these plots assuming quasi-equilibrium.

Constant	True value	$(k_{+s}[S] + k_{-s}) \gg k_{-1}$.	
		Value assuming quasi-equilibrium	
pK_1	8.00	8.00	
pK'_1	8.00	8.00	
pK_2	4.00	4.00	
pK'_2	$-\infty$	$-\infty$	
k_{-s}	$10^4 s^{-1}$	—	
k_{+s}	$10^6 s^{-1} \cdot M^{-1}$	—	
k	$10^5 s^{-1}$	$10^5 s^{-1}$	
K_s	$10^{-2} M$	$1.1 \times 10^{-1} M$	
K_m	$1.1 \times 10^{-1} M$	$1.1 \times 10^{-1} M$	

cations inherent in this set of results are mentioned below.

Whenever steady-state rather than quasi-equilibrium obtains for an enzymic system, the interpretation of initial rate data becomes problematic. And it is not easy to rule out steady-state kinetics unequivocally from such data.

(4) The test suggested by Haldane (1930) and by Morales (1955), according to which non-competitive interaction (i.e. large changes in $V_{(app.)}$ without a reasonable change in $K_{m(app.)}$) would indicate the independence of $V_{(app.)}$ and $K_{m(app.)}$ and hence quasi-equilibrium, is not necessarily valid. This is illustrated in Fig. 4, drawn from eqn. (18), where it can be seen that at high pH $\log V_{(app.)}$ decreases with decreasing H^+ ion concentration whereas $K_{m(app.)}$ is independent of the H^+ ion concentration, although this is a steady-state and not a quasi-equilibrium situation.

It therefore appears that in the systems discussed above (all of them characterized by the fact that the ionization of those groups at the active site which are still able to ionize in the enzyme-substrate complex are the same as in the free enzyme), the criterion by which quasi-equilibrium and steady state can be differentiated in experiments in which initial rates are measured, rests on the property of apparent substrate activation of the steady-state systems. Such apparent substrate activation will depend on

the relative values of various rate constants, as has been described above, so that it may actually be absent. If present, it will be restricted to certain ranges of H^+ concentration (since, for example, whenever $[H^+] \gg K_1$ and K_2 or when:

$$K_2 \gg [H^+] \gg K_1$$

Lineweaver-Burk plots will be linear).

From the foregoing discussion it should be clear that some care is required in the interpretation of both substrate-activation effects and in the assignment of values to dissociation constants of ionizing groups in the active site of enzymes.

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