SOME ASPECTS OF THE KINETICS OF ENZYMIC REACTIONS

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INTRODUCTION

An interpretation of the variation of the rate of enzymic reactions with pH is necessary to determine the relationship between the observed (pH-dependent) and the true (pH-independent) velocity constants. This relationship is particularly important when the rate is being studied at different temperatures in order to determine thermodynamic quantities. The accounts of the effect of pH on the rate of enzymic reactions given in reviews[8,10,20,23,26,29] have not considered detailed kinetic schemes based on the presence of the enzyme in several charged forms of differing reactivity. In the discussion presented below these kinetic schemes are found to arise from a simple general treatment which includes the special cases previously examined[7,27,28]. This paper also includes some brief comments on the basis of the equations used and on the interpretation of the observed velocity constants.

THE MICHAELIS-MENTEN EQUATION

The intervention of a complex (ES) in the decomposition of the substrate (S) to products (P) catalysed by an enzyme (E) was postulated by MICHAELIS AND MENTEN[18]:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_3} E + P.$$

The differential equations resulting from this scheme have been solved by CHANCE[4] who was able to determine directly the concentration of the complex in several cases. In general, however, the concentration (y) of the complex cannot be measured and it is assumed that the approximation of the stationary state method applies. BRIGGS AND HALDANE[1] have pointed out that since the concentration of the enzyme is usually low compared to that of the substrate, the *average* rate of change of the concentration of the complex must be much less than that of the substrate when the concentration of the complex is decreasing. The same relationship may be shown to hold for the instantaneous rates of change. If E_o is the total concentration of the enzyme, then

$$\begin{aligned} dy/dt &= k_1(E_o - y)[S] - (k_2 + k_3)y, \\ -d[S]/dt &= k_1(E_o - y)[S] - k_2 y \end{aligned} \qquad (1)$$

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Thus, putting $d^2y/d[S]^2 = 0$ gives the maximum value of $dy/d[S]$ as

$$dy/d[S] = y(E_o - y)/E_o[S].$$

If $E_o \ll [S]$, $dy/dt \ll d[S]/dt$, and since the numerical value of $d[S]/dt$ is less than $k_1E_o[S]$, it follows that $dy/dt \ll k_1E_o[S]$ so that the term dy/dt in equation (1) may be neglected and we may write

$$y = E_o[S]/(K_m + [S])$$
$$\text{and } -d[S]/dt = k_3E_o[S]/(K_m + [S]), \quad (2)$$

where $K_m = (k_2 + k_3)/k_1$.

Determination of velocity constants. The parameters K_m and k_3E_o can be obtained from rearranged forms of equation (2) as described by LINEWEAVER AND BURKE[14]; it will be seen that, on the basis of the equations deduced below, either or both of the parameters so determined may be functions of the hydrogen ion concentration. Although k_3 is thus found directly, the value of K_m does not enable either k_2 or k_1 (or their ratio) to be determined; K_m will approximate to k_2/k_1, or k_3/k_1, if k_2 is either much larger or much smaller than k_3. The relative magnitudes of k_2 and k_3 cannot be predicted, nor can they directly be found from a study of the rate of a reaction followed only by the disappearance of the substrate or the formation of the products. Thus the change of K_m with temperature gives no unequivocal evidence about the magnitude of the change in thermodynamic quantities resulting from formation of the enzyme-substrate complex. Change of the medium (*e.g.* ordinary to heavy water) makes it possible to compare the resulting changes in K_m and k_3[24], but it is still necessary to make quite arbitrary assumptions about the changes in k_1 and k_2 before a decision can be reached as to whether k_2 is considerably larger or smaller than k_3[15]. The pH-variation of the apparent values of K_m and k_3 has also been used in this connection; the case of invertase was cited[8]: in the alkaline region although k_3 varies with the pH, K_m does not. Hence, it was concluded $k_2 \gg k_3$. But equation (12), deduced below, predicts this behaviour for the apparent values of K_m and k_3, and so, if our assumptions are valid, no deductions can be drawn as to the relative magnitude of k_2 and k_3. In the special case of peroxidases and catalases CHANCE[5] has been able to determine these velocity constants directly; k_2 is found to be negligible, and k_1 is large (10^3 to 10^7 l mol^{-1}sec^{-1}). It should be noted that a lower limit to k_1 can be obtained from K_m and k_3, and the formation of a complex between the enzyme and substrate is frequently a rapid reaction, *e.g.* with such typical values as $K_m = 10^{-3}$ mol/l and $k_3 = 10$ sec^{-1}, k_1 must be greater than 10^4 l mol^{-1}sec^{-1}.

Determination of velocities. Since it is common for the products of an enzymic reaction to act as inhibitors, and since some enzymes do not retain their full activity throughout the course of the reaction, it is usual to measure the initial velocity; this may conveniently be done by the secant method[30]. The amount of substrate decomposed in a given time is a useful measure of the reaction velocity for systems obeying zero-order kinetics. This method is, however, frequently used even when the system does not obey zero-order kinetics, the velocity so found then being used to find K_m and k_3E_o. The values obtained in this way will clearly differ from the true values by an amount depending on the constants themselves, and on the extent of reaction. BODANSKY[2] stressed the obvious point that some functions of the amounts changed in a given time, rather than the amounts themselves, should be used. The nature of the function to be

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used when equation (2) holds has been pointed out by WALKER AND SCHMIDT[27]. The integrated form of equation (2) may be written

$$x = k_3 E_0 t + K_m \log(1 - x/S_0), \tag{3}$$

where S_0 is the initial substrate concentration and x the amount of substrate decomposed after the fixed time t; thus a plot of x against $\log(1 - x/S_0)$ will be linear, with gradient K_m and intercept $k_3 E_0 t$.

It should be noted that when the rate is being measured at various pH's, the ionic strength of the reaction mixture must be kept constant. If the ionic strength varies appreciably the ionisation constants will alter, and so will the velocity constants if the reactants are charged.

THE VARIATION OF RATE WITH pH

To begin with we will assume that the effect of pH is only on the enzyme; this will be true if the substrate is uncharged, or does not change appreciably its state of charge in the range considered (*e.g.* a carboxyl group of pK 3 at pH's greater than 6), and may be true if, kinetically, there is no distinction between different charged species of the substrate. And the effect of pH on the enzyme will be limited further by considering only three species, written as E, HE and H_2E^+; although these will be (for the sake of convenience) referred to as the negatively charged, uncharged and positively charged forms of the enzyme they may be any three kinetically significant forms represented by the equilibria:

$$HE \rightleftarrows H^+ + E^-$$
$$H_2E^+ \rightleftarrows H^+ + HE$$

The ionisation constants are then defined:

$$K_a = [H^+]\,[E^-]/[HE],\ K_b = [H^+]\,[HE]/[H_2E^+].$$

On these assumptions, there are several possibilities: the substrate may combine with any one, two or all three of the charged forms of the enzyme, and one or more forms of the complex may then undergo decomposition; this last range of possibilities is usually restricted by the observation of an optimum pH. A few examples will suffice to illustrate how equations may be deduced based on some of these possibilities. If we assume that the substrate combines with all three forms of the enzyme, but that only the uncharged form of the complex undergoes decomposition, then as well as the equations given above for the ionisation of the enzyme we have:

$$S + HE \underset{k_2}{\overset{k_1}{\rightleftarrows}} S.HE \xrightarrow{k_3} HE + P$$
$$HES \rightleftarrows H^+ + ES \tag{4}$$
$$H_2ES^+ \rightleftarrows H^+ + HES \tag{5}$$

$$\text{If } K_c = [H^+]\,[ES^-]/[HES],\ K_d = [H^+]\,[HES]/[H_2ES^+],$$

and if the total enzyme concentration is E_0 and h is written for $[H^+]$, then

$$[HE]\,(1 + h/K_b + K_a/h) + [HES]\,(1 + h/K_d + K_c/h) = E_0,$$

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and, using the stationary state equation

$$k_1[S][HE] - (k_2 + k_3)[HES] = 0$$

to solve for [HES], we obtain

$$[HES] = \frac{E[_oS]}{[S](1 + h/K_d + K_c/h) + K_m(1 + h/K_b + K_a/h)}$$

Thus if the rate of disappearance of substrate at this pH, $(-\delta[S]/\delta t)_h$, be written as R,

$$R = \frac{k_3E_o[S]}{[S](1 + h/K_d + K_c/h) + K_m(1 + h/K_b1 + K_a/h)} \quad (6)$$

To avoid having to disentangle the effect of two variables ([S] and h) on the rate it is usual to measure the initial rate at various pH's at a constant initial substrate concentration, S_o; equation (6) becomes

$$R = \frac{k_3E_oS_o}{S_o(1 + h/K_d + K_c/h) + K_m(1 + h/K_b + K_a/h)} \quad (7)$$

Equation (7) was deduced assuming that the decomposition of the complex is not catalysed by hydrogen or hydroxyl ions; such catalysis is inconsistent with a scheme in which only the uncharged form of the complex undergoes decomposition if the resulting rate equation is to have a pH-optimum.

The reaction of the substrate with the charged forms of the enzyme does not have to be considered explicitly; the concentration of the charged complexes (ES^- and H_2ES^+) will be controlled by the equilibria (4) and (5) when the proton-transfer reactions are the fastest ones present.

Before considering equation (7) further the simpler cases will be considered in which the substrate combines with only one or two forms of the enzyme. If the substrate combines only with the uncharged form of the enzyme, equation (7) becomes

$$R = \frac{k_3E_oS_o}{S_o + K_m(1 + h/K_b + K_a/h)} \quad (8)$$

The pH optimum is found by solution of $(\delta R/\delta h)_{S_o} = 0$, (since h is not zero, the h optimum and the pH optimum are the same) giving $h = (K_aK_b)^{1/2}$; the rate at this point, R_{opt}, is then

$$R_{opt} = \frac{k_3E_oS_o}{K_m[1 + 2(K_a/K_b)^{1/2}] + S_o} \quad (9)$$

Now, by definition, K_a is less than K_b; if K_a is much less than K_b, equation (9) becomes

$$R_{opt} = k_3E_oS_o/(K_m + S_o) \quad (10)$$

and the apparent MICHAELIS constant is the same as the true one; at other pH's the apparent constant is greater.

A particular feature of this case is that the MICHAELIS constant is the only term in equation (8) which is pH-dependent. If the reaction is zero-order over the entire pH-range investigated it cannot be interpreted according to this scheme, which

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predicts no variation of rate with pH for a zero-order reaction. This is because in a zero-order reaction essentially all the enzyme is combined with the substrate to give (we have assumed) an active complex, and alteration of the pH cannot change the rate as long as the substrate competes with overwhelming success for the uncharged form (HE) of the enzyme. The reaction may, of course, appear to be zero-order at the pH-optimum but not at other pH values.

An example of a reaction interpreted according to this scheme is provided by acetylcholine esterase; in this case there is the added complication of inhibition by the substrate, but, leaving this out of account here, WILSON AND BERGMANN[28] showed that

$$R_{opt}/R = 1 + ah/K_b + aK_a/h, \tag{11}$$

where $a = K_m/(K_m + S_o)$; this follows from equations (8) and (10). The constants K_a and K_b were found from equation (11) by plotting R_{opt}/R against h on the acid side of the pH-optimum, and against $1/h$ on the alkaline side.

We will now consider the case in which the substrate combines with both the uncharged and negatively charged forms of the enzyme, so that equation (7) becomes

$$R = \frac{k_3 E_o S_o}{S_o(1 + K_c/h) + K_m(1 + h/K_b + K_a/h)} \tag{12}$$

Here the pH optimum depends on the substrate concentration, and is given by

$$h_{opt} = [K_b(K_a K_m + K_c S_o)/K_m]^{1/2}.$$

If we write $K_c = \beta K_a$, the rate at the pH optimum is

$$R_{opt} = \frac{k_3 E_o S_o}{K_m + S_o + 2[K_a K_m(K_m + \beta S_o)/K_b]^{1/2}}$$

and hence

$$\frac{R_{opt}}{R} = \frac{1 + 2\,[K_a K_m/K_b(K_m + \beta S_o)]^{1/2} + S_o(1 - \beta)/(K_m + \beta S_o}{1 + K_a/h + K_m h/K_b(K_m + \beta S_o + S_o(1 - \beta)/(K_m + \beta S_o)} \tag{13}$$

An example of this class is invertase[12,17,19]; it has been found that the alkaline part of the plot of R/R_{opt} against pH is independent of the substrate concentration, and is expressed by

$$R/R_{opt} = 1/(1 + K_a/h),$$

and this follows from equation (13) when $K_a/K_b \ll 1$ (as is the case for invertase) and β is close to 1, *i.e.* the ionisation constant of the enzyme is not appreciably altered by complex formation.

When the substrate combines with all the three forms of the enzyme, equation (7) applies. If we write $K_c = \beta K_a$, $K_d = K_b/\gamma$, the pH optimum is given by

$$h_{opt} = \left\{K_a K_b \left[1 + \frac{(\beta - \gamma)S_o}{\gamma S_o + K_m}\right]\right\}^{1/2}$$

and thus

$$\frac{R_{opt}}{R} = \frac{K_m + S_o + (\gamma S_o + K_m)h/K_b + (\beta S_o + K_m)K_a/h}{K_m + S_o + 2[(\beta S_o + K_m)(\gamma S_o + K_m)K_a/K_b]^{1/2}}$$

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If we assume that the ionisation constants of the enzyme are the same if it is free or combined with the substrate, $\beta = \gamma = 1$, and

$$R = k_3 E_o S_o / (1 + h/K_b + K_a/h)\ (K_m + S_o), \tag{14}$$

and hence

$$\frac{R_{opt}}{R} = \frac{1 + h/K_b + K_a/h}{1 + 2(K_a/K_b)^{1/2}} \tag{15}$$

In equation (14) only the apparent k_3 is pH-dependent, and equation (15) shows that the variation of the R_{opt}/R with pH should be independent of the substrate and depend only on the enzyme. The decomposition of histidine by histidase has been interpreted along these lines[27]. The experiments were carried out at a histidine concentration such that the reaction was zero-order, and the fall in rate in the acid region was explained by the complex gaining a proton; the alkaline region was not interpreted quantitatively, but the points up to pH 10 can be accounted for by equation (15), assuming $pK_a = 10$.

Ionisation of the substrate. This introduces many further possibilities; only one case will be considered. The substrate (total concentration S_o) will be assumed to be present in two forms, SH and S^-, the equilibrium between them being

$$SH \rightleftarrows S^- + H^+$$

and the ionisation constant K_s. For the enzyme we shall now only consider one equilibrium

$$H_2E^+ \rightleftarrows H^+ + HE.$$

This simplification is used since it is normally only possible to determine two ionisation constants from the variation of rate with pH at one substrate concentration. Assuming that combination takes place only between SH and HE, it may readily be shown that

$$R = \frac{k_3 E_o S_o}{(1 + K_s/h)\ (1 + h/K_b) K_m + S_o} \tag{16}$$

Also $h_{opt} = (K_s K_b)^{1/2}$, and hence

$$R_{opt} = \frac{k_3 E_o S_o}{[1 + (K_s/K_b)^{1/2}]^2 K_m + S_o} \tag{17}$$

The decomposition of arginine by arginase has been interpreted by an equation similar to equation (16)[7]; the apparent Michaelis constant was found to vary with pH, there being a minimum at $h = (K_s K_b)^{1/2}$. The results, however, show a variation of the apparent k_3 with pH, and this is not predicted by equation (16); although an equation of the right form may be obtained by assuming that both forms of the enzyme combine with one form of the substrate, the results apparently can still not be accounted for quantitatively.

Some of the other rate equations that may be deduced on the basis that two charged forms of the enzyme are operative resemble equations in which the ionisation of one of the enzymic species is replaced by ionisation of the substrate. It is not usually possible to discriminate between similar equations such as (16) and (8) on a kinetic

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basis alone, and a decision must therefore be based on experiments using several substrates, or an independent knowledge of the ionisation constants of the substrate.

CONCLUSION

The effects of pH on the rate, as considered here, have a bearing on the conclusions drawn from enzymic reactions carried out at varying temperatures. Thus heats and entropies of activation are customarily deduced without considering the contributions made by heats and entropies of ionisation[3,11,13]. However in certain cases when the reaction is carried out at the pH-optimum this may be justified, *i.e.* when the true and apparent values of K_m and k_3 are the same (*cf.* equations (9) and (10)).

It should be noted that the so-called "uncharged" form of the enzyme (written as HE) is not taken to be identical with the form with minimum net charge and so there is no reason why the pH optimum should coincide with the iso-electric point. There may, indeed, be no connection between the ionic properties of the protein molecule as a whole and those of the "active centres" responsible for enzymic activity. Although inactivation of the enzyme has not been explicitly considered, it will not alter the equations deduced above if the rate of inactivation is low compared to the rate of reaction.

The simple treatment present here is obviously only a first approximation, perhaps only applicable in a few cases, but it leads to equations which are readily tested. The theory of MICHAELIS AND MENTEN involving an enzyme-substrate complex can account for the variation of rate with pH, and is thereby confirmed, as it also is by interpretations of inhibition phenomena (MASSART, 1951). The ionisation constants found may help to elucidate the nature of the "active centre" of the enzyme, particularly in conjunction with experiments in which the enzyme has been chemically altered without destroying its activity[6,9,25]. It is interesting that each of the enzymes taken as examples (acetylcholine esterase, invertase, histidase and arginase) is found by the methods described above to have a pK close to 7. The two kinds of groups in proteins which normally have a pK of about 7 are iminazoles, and amino groups at the ends of polypeptide chains. The data, however, are not sufficiently extensive to warrant any more detailed speculation.

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SUMMARY

1. The basis of the MICHAELIS-MENTEN equation, and the determination of velocity constants from it, are discussed.

2. A simple scheme for interpreting the effect of pH on the rate of enzymic reactions is presented, and its application illustrated by examples.

RÉSUMÉ

1. La base de l'équation de MICHAELIS-MENTEN et la détermination, des constantes de vitesse à partir de cette dernière, sont discutés.

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2. L'auteur présente un schéma simple pour l'interprétation de l'effet du pH sur la vitesse des réactions enzymatiques; il donne des exemples d'application de ce schéma.

ZUSAMMENFASSUNG

1. Die Grundlage der MICHAELIS-MENTEN'schen Gleichung und die Bestimmung der Geschwindigkeitskonstanten mit Hilfe dieser Gleichung werden erörtert.

2. Ein einfaches Schema zur Auslegung der Wirkung des pH auf die Geschwindigkeit enzymatischer Reaktionen wird angegeben und seine Anwendung an Hand von Beispielen erklärt.

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