ON THE INTERPRETATION OF THE pH VARIATION OF THE MAXIMUM INITIAL VELOCITY OF AN ENZYME-CATALYZED REACTION

by

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INTRODUCTION

Knowledge of the dissociation constants and heats of ionization of groups at the active center of an enzyme would provide valuable information for the understanding of enzyme activity. DIXON[1] has recently described methods by which the ionization constants of groups at the active center may be obtained from the variation of Michaelis constants and inhibition constants with pH. The purpose of this article is to discuss how similar information may be obtained from the variation of maximum initial velocity with pH.

It is generally found that there is an optimum pH or range of pH for an enzyme-catalyzed reaction. In more acidic or basic solutions the activity diminishes toward zero. MICHAELIS AND DAVIDSOHN[2] and MICHAELIS AND PECHSTEIN[3] suggested that this reversible loss of catalytic activity may result from ionization of acidic and basic groups of the enzyme which affect the reaction. If this is the case the plot of maximum initial velocity versus pH must be a symmetrical bell-shaped curve as represented by equation (6) below, and by use of this equation the ionization constants of the two groups may be obtained from the experimental data.

As discussed by JOHNSON[4] it is important to distinguish between loss of activity resulting from denaturation of the enzyme by acid and base and reversible loss of activity. Our considerations will be restricted to reversible effects and, therefore, only apply to experiments from which irreversible losses of activity have been eliminated. Variation of rate with pH may also result from ionization of the substrate[5,6,7], but such ionization is omitted from the present treatment although it could be readily introduced.

Since the interpretation of the pH variation of the velocity at any particular substrate concentration is more involved than the interpretation of the maximum initial velocity obtained by extrapolation[8] to infinite substrate concentration, the following treatment will be restricted to interpretation of the maximum initial velocity. In some cases the maximum initial velocity may be closely approximated by initial velocities at some high substrate concentration, provided that substrate inhibition or activation does not occur.

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The ionization of two groups of the enzyme which are essential for activity is represented by

$$E^{n-1} \underset{}{\overset{K_{aE}}{\rightleftharpoons}} H^{+} + E^{n} \qquad (1)$$

$$E^{n} \underset{}{\overset{K_{bE}}{\rightleftharpoons}} H^{+} + E^{n+1} \qquad (2)$$

where E^n is the catalytically-active form, and K_{aE} and K_{bE} are acid dissociation constants for the two groups which may be any type of ionizable groups, that is, equation (2) does not necessarily represent the ionization of a "basic" group. Since there is no reason to believe that the charge on the enzyme or even the active site will be zero for the active form, a noncommittal n is used to represent the net negative charge of the active form. Equations (1) and (2) represent the net negative charge of the active form. Equations (1) and (2) represent the simplest hypothesis which will account for the loss of activity in both acidic and basic solutions, but it is to be expected that a larger number of ionizable groups may play an important role in many cases.

The combination of S with E^n and the subsequent dissociation of product P are represented by

$$E^{n} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E^{n}S \xrightarrow{k_3} E^{n} + P \qquad (3)$$

If reactions (1)–(3) are the only ones considered it may be shown[7] that the maximum initial velocity will be independent of pH. This results since at an infinite concentration of substrate the equilibria are displaced so that all of the enzyme is in the form E^nS at any hydrogen ion concentration. Therefore, it is necessary to allow in addition for the dissociation of the two ionizable groups in the enzyme-substrate complex.

The total mechanism is represented by

$$\begin{tikzcd}
E^{n-1} \arrow[r, shift left, "K_{aE}"] & E^{n} \arrow[l, shift left] \arrow[r, shift left, "K_{bE}"] \arrow[d, shift right, "k_1"'] & E^{n+1} \arrow[l, shift left] \\
E^{n-1}S \arrow[r, shift left, "K_{aES}"] & E^{n}S \arrow[l, shift left] \arrow[r, shift left, "K_{bES}"] \arrow[u, shift right, "k_2"'] \arrow[d, "k_3"] & E^{n+1}S \arrow[l, shift left] \\
 & E^{n} + P &
\end{tikzcd} \qquad (4)$$

This mechanism is the same as the most general one discussed by WALEY[7] who has given the steady-state treatment for the case that the hydrogen ion equilibria are adjusted rapidly and $(S) \gg (E)_0$, where $(E)_0$ is the total molar concentration of the enzymically active sites. The result of this steady-state treatment may be expressed in terms of the usual MICHAELIS[9] equation for the initial velocity v

$$v = \frac{V}{1 + K_m/(S)} \qquad (5)$$

where

$$V = \frac{k_3(E)_0}{1 + (H^+)/K_{aES} + K_{bES}/(H^+)} \qquad (6)$$

$$K_m = \frac{(k_2 + k_3)}{k_1} \cdot \frac{1 + (\mathrm{H}^+)/K_{a\mathrm{E}} + K_{b\mathrm{E}}/(\mathrm{H}^+)}{1 + (\mathrm{H}^+)K_{a\mathrm{ES}} + K_{b\mathrm{ES}}/(\mathrm{H}^+)} \tag{7}$$

DIXON[1] has derived an equation for the pH variation of K_m which is equivalent to equation (7) and has discussed the determination of the various ionization constants from experimental data.

Before proceeding to the discussion of the determination of the constants in equation (6) it is appropriate to make some general remarks about this mechanism. The fact that the equilibria $\mathrm{E}^{n-1} + \mathrm{S} \rightleftharpoons \mathrm{E}^{n-1}\mathrm{S}$ and $\mathrm{E}^{n+1} + \mathrm{S} \rightleftharpoons \mathrm{E}^{n+1}\mathrm{S}$ are not indicated is not because they are assumed to be lacking but because the concentrations of E^{n-1}, E^{n+1}, $\mathrm{E}^{n-1}\mathrm{S}$ and $\mathrm{E}^{n+1}\mathrm{S}$ will always be determined by the concentrations of E^{n}, $\mathrm{E}^{n}\mathrm{S}$, and (H^+) if the hydrogen ion equilibria are assumed to be adjusted rapidly in comparison with the steps of reaction (3). Although $\mathrm{E}^{n-1}\mathrm{S}$ and $\mathrm{E}^{n+1}\mathrm{S}$ might be allowed to yield product at slower rates than $\mathrm{E}^{n}\mathrm{S}$, the fact that zero activity is generally approached in acidic and basic solutions indicates that such reactions may be ignored. If these complexes did yield product then asymmetrical activity—pH curves would result, and the extent of asymmetry would be related to the relative rates of breakdown of the three enzyme-substrate complexes.

It is worth considering what the relationship between $K_{a\mathrm{E}}$ and $K_{a\mathrm{ES}}$ or $K_{b\mathrm{E}}$ and $K_{b\mathrm{ES}}$ may be. If the substrate is an ion its electrostatic effect on the ionization of groups in the vicinity of the binding site may be predicted. A negatively charged substrate molecule will cause the protons of the nearby acid groups to be bound more strongly so that $K_{a\mathrm{E}} > K_{a\mathrm{ES}}$ and $K_{b\mathrm{E}} > K_{b\mathrm{ES}}$. On the other hand, a positively charged substrate molecule will exert an acid-strengthening effect.

CALCULATION OF IONIZATION CONSTANTS FROM PLOTS OF MAXIMUM INITIAL VELOCITY VERSUS pH

Equation (6) represents the bell-shaped plot of V versus pH which is frequently obtained experimentally. A series of plots for $K_{a\mathrm{ES}} = 10^{-6}$ and a variety of values of $K_{b\mathrm{ES}}$ are given in Fig. 1 for $k_3(\mathrm{E})_0 = 1$. It is evident that when the curve is broad

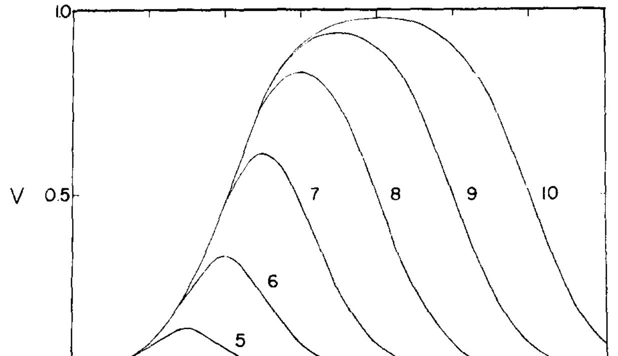

Fig. 1. Variation of maximum initial velocity with pH according to eqn. (6) with $k_3(\mathrm{E})_0 = 1$, $\mathrm{p}K_a = 6.00$ and for values for $\mathrm{p}K_b$ given on the graph.

the pK's of the two groups may be read off as the pH's at which the velocity has half its maximum value. However, in the case in which the two ionizations are not well separated this method fails, and it is necessary to use a different approach.

The relationship between the pH of maximum activity and K_a and K_b (these symbols being used in the following discussion for $K_{a\mathrm{ES}}$ and $K_{b\mathrm{ES}}$) may be obtained by setting the first derivative of equation (6) equal to zero. This yields

$$(\mathrm{H}^+)^2_{\max} = K_a K_b \tag{8}$$

or

$$\mathrm{pH}_{\max} = 1/2(\mathrm{p}K_a + \mathrm{p}K_b)$$

which formally resembles the equation for the isoelectric point of a dipolar ion. The maximum initial velocity at this pH is

$$V_{\max} = \frac{k_3(\mathrm{E})_0}{1 + 2\sqrt{K_b/K_a}} \tag{9}$$

so that equation (6) may be written as

$$V = \frac{V_{\max}(1 + 2\sqrt{K_b/K_a})}{1 + (\mathrm{H}^+)/K_a + K_b/(\mathrm{H}^+)} \tag{10}$$

If $(\mathrm{H}^+)_a$ is the hydrogen ion concentration at which $V/V_{\max} = 1/2$ on the acid side and $(\mathrm{H}^+)_b$ is the hydrogen ion concentration at the corresponding point on the basic side, it may be shown from equation (10) that

$$K_a K_b = (\mathrm{H}^+)_a(\mathrm{H}^+)_b \tag{11}$$

Substituting into (10) yields

$$K_a = (\mathrm{H}^+)_a + (\mathrm{H}^+)_b - 4\sqrt{(\mathrm{H}^+)_a(\mathrm{H}^+)_b} \tag{12}$$

or introducing (8),

$$K_a = (\mathrm{H}^+)_a + (\mathrm{H}^+)_b - 4\,(\mathrm{H}^+)_{\max} \tag{13}$$

Thus by use of equation (12) or (13) the ionization constant of group a may be calculated even when the pK's are not well separated. The ionization constant of group b may then be calculated by use of equation (8) or (11). However, as pK_b becomes smaller the error in the calculated value of pK_a which results from a given error in pH$_a$ and pH$_b$ increases. This is illustrated by the data in Table I which gives pH$_a$ and pH$_b$ for a series of pK_b values with pK_a held constant at 6.00. The error in pK_a resulting from the experimental value of the pH of half maximum velocity on the acidic side being 0.05 too low and the pH at half maximum velocity on the basic side being 0.05 too high is given in the last column. Thus it is seen that if $\mathrm{p}K_b < \mathrm{p}K_a$ extremely accurate experimental data would be required to obtain very accurate pK's for the two ionizable groups. This results from the fact that if $\mathrm{p}K_b \ll \mathrm{p}K_a$, the shape of the curve is nearly independent of the pK's, that is, a rather large change in pK_a or pK_b has a rather small effect on the width of the peak. The preceding column (Δ pH) which gives the widths of the plots at the half maximum velocity shows that as pK_b decreases the curves become narrower, and so the width of the plots is a useful guide in indicating the relative magnitudes of pK_a and pK_b. The width of the curve depends only upon $(\mathrm{p}K_b - \mathrm{p}K_a)$. Thus the width is the same for $\mathrm{p}K_a = 7$ and $\mathrm{p}K_b = 6$ as for $\mathrm{p}K_a = 6$ and $\mathrm{p}K_b = 5$.

TABLE I

PROPERTIES OF PLOTS OF MAXIMUM INITIAL VELOCITY (V) VERSUS pH FOR $pK_a = 6.00$

pK_b	pH_a	pH_b	ΔpH	Error in pK_a*
10	5.98	10.02	4.04	0.05
9	5.95	9.05	3.10	0.06
8	5.86	8.14	2.28	0.07
7	5.65	7.35	1.70	0.10
6	5.32	6.68	1.36	0.20
5	4.89	6.11	1.22	0.40
4	4.41	5.58	1.17	0.73
3	3.92	5.08	1.16	1.17
2	3.43	4.57	1.14	1.65

* Difference between the correct value of pK_a (6.00) and that resulting from pH_a being 0.05 too low and pH_b being 0.05 too high.

Once K_a and K_b have been determined in this way, a test of the applicability of this theory is whether or not equation (6) represents the data over the whole range of pH.

THE EFFECT OF BUFFER IONS ON THE MAXIMUM INITIAL VELOCITY

If the concentration or nature of the buffer has an effect on the maximum initial velocity of the enzymic reaction at a given pH the interpretation of the results may not be as simple as that outlined above. In order to account for buffer effects it is necessary to include buffer-enzyme equilibria in the mechanism[10]. However, there is one simple case in which the form of equation (6) is unchanged, and only the interpretation of $K_{a\mathrm{ES}}$ and $K_{b\mathrm{ES}}$ is altered. That is the case in which the buffer-binding sites which affect the maximum initial velocity become saturated at a sufficiently low buffer concentration so that the enzyme may be studied under conditions such that the buffer-binding sites are saturated, that is, further increasing the concentration of the buffer produces no further effects on the kinetics. When this is the case the ionization constants determined by use of equation (6) will be those for enzyme saturated with buffer as well as substrate.

Asymmetrical plots of maximum initial velocity versus pH may result from the use of different buffer systems in various pH ranges if there are appreciable interactions of the enzyme with components of the buffer. If the enzyme interacts with anions, the effect of changing buffer systems can be greatly reduced by using uncharged-base type buffers like *tris*-(hydroxymethyl)-aminomethane since the concentration of a given anion can be held constant over a wide range of pH.

In the case of buffers like phosphate there is the possibility that at low pH values the ionizable groups of the enzyme may be affected by bound $H_2PO_4^-$ ions while at high pH values ionizable groups are affected by bound HPO_4^{-2} ions. However, the affinity of the enzyme for the two ions may be very different so that divalent ions are bound predominately even at low pH where the monovalent ions are more abundant (or vice versa). Thus in the case of a buffer like phosphate it is impossible to determine from this type of experiment whether the values of pK's obtained are those for enzyme binding $H_2PO_4^-$ ions, HPO_4^{-2} ions or each a certain fraction of the time.

DISCUSSION

It is apparent from equation (6) why the pH optimum for an enzyme may be different for the forward and reverse reactions which are catalyzed. This is because the ionization constants which are of importance in determining the maximum initial velocity are those for the enzyme-substrate complex, and there is no reason why these should be identical for different substrates. However, in the case of an enzyme which follows mechanism (4) the dissociation constants for the free enzyme may be determined by plotting V/K_m versus pH since

$$\frac{V}{K_m} = \frac{k_1 k_3 (\mathrm{E})_0/(k_2 + k_3)}{1 + (\mathrm{H}^+)/K_{a\mathrm{E}} + K_{b\mathrm{E}}/(\mathrm{H}^+)} \qquad (14)$$

and the methods described earlier may be used for the calculation of $K_{a\mathrm{E}}$ and $K_{b\mathrm{E}}$. As in the case of equations (6) and (7) this equation is strictly applicable only if any interaction of buffer with the enzyme is without effect on the activity.

In favourable cases it should not only be possible to determine the pK's for the ionizable groups essential for enzymic activity but also the heat of ionization from studies of maximum initial velocity at a series of temperatures. With information as to both pK and Δ H for ionizing groups in enzymes it should be possible to identify the ionizable groups in the vicinity of the active site with some certainty as has been done in the case of heme-linked acid groups for hemoglobin[11].

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SUMMARY

1. The variation of the maximum initial velocity for an enzymic reaction with pH is discussed in terms of the dissociation of two acid groups which affect the enzymic activity.

2. The effect of ionic substrates upon the pK's of these groups is discussed.

3. A method for calculating the pK's of the ionizable groups from plots of maximum initial velocity versus pH is developed.

4. The effect of interaction between components of the buffer and the enzyme upon the values of the pK's is discussed.

5. A method is suggested for the determination of the pK's of the ionizing groups in the free enzyme, in contrast to those in the enzyme-substrate complex.

RÉSUMÉ

1. La variation de la vitesse initiale maximum d'une réaction enzymatique en fonction du pH peut être rapprochée de la dissociation de deux groupes acides qui jouent un rôle dans l'activité enzymatique.

2. L'influence de substrats ionisables sur les pK's de ces groupes est discutée.

3. Les auteurs ont mis au point une méthode de calcul des pK's des groupes ionisables à partir des courbes représentant la variation de la vitesse initiale maximum en fonction du pH.

4. L'influence de l'interaction entre les constituants du tampon et l'enzyme sur les valeurs des pK's est discutée.

5. Les auteurs proposent une méthode de détermination des pK's des groupes ionisables dans l'enzyme libre, en opposition aux pK's dans le complexe enzyme-substrat.

ZUSAMMENFASSUNG

1. Für die Abhängigkeit der maximalen Anfangsgeschwindigkeit einer enzymatischen Reaktion vom pH wird die Dissoziation von zwei Sauregruppen, die die enzymatische Aktivität beeinflussen, verantwortlich gemacht.

2. Die Wirkung der ionischen Substrate auf die pK's dieser Gruppen wird besprochen.

3. Eine Methode zur Berechnung der pK's der ionisierbaren Gruppen durch Auftragen der maximalen Anfangsgeschwindigkeit gegen das pH wird entwickelt.

4. Die Wirkung der Reaktion zwischen den Bestandteilen des Puffers und des Enzyms auf die pK-Werte wird besprochen.

5. Es wird eine Methode vorgeschlagen zur Bestimmung der pK's der ionisierenden Gruppen des freien Enzyms im Gegensatz zu denen des Enzym-Substrat-Komplexes.

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