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Kinetic Behaviour of Calf-Intestinal Alkaline Phosphatase with 4-Methylumbelliferyl Phosphate

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1. The effects of varying pH, ionic strength and temperature on the parameters K_m and $V_{max.}$ for a purified alkaline phosphatase from calf intestinal mucosa with a new fluorogenic substrate, 4-methylumbelliferyl phosphate monoester disodium salt, and an ammediol–hydrochloric acid buffer system were determined. 2. It was found that, under varying conditions, a relationship exists between K_m and $V_{max.}$ such that $V_{max.} = \beta/(1+\alpha/K_m)$, where α and β are constants, temperature- and ionic strength-dependent, but pH-independent. It is shown that this relationship accounts satisfactorily for the well-known effect of varying substrate concentration on optimum pH and velocity. 3. The various results are interpreted in terms of a pH-dependent conformational equilibrium between two forms of the enzyme, E_1 and E_2. Only E_1 combines with substrate, and only E_2 reacts to give inorganic phosphate. 4. To account for the pH-variation of K_m and $V_{max.}$ in terms of this theory, it is postulated that the conformational change is associated with a change in pK of two basic groups in the enzyme.

Ross, Ely & Archer (1951) observed that a linear relationship exists between the logarithm of the substrate (β-glycerophosphate) concentration and the optimum pH for hydrolysis with rat-intestinal-mucosal alkaline phosphatase. A second linear relationship, between the reciprocals of substrate (phenyl phosphate) concentration and optimum velocity of hydrolysis with varying pH, was found by Morton (1957). The former relationship has been used (Motzok & Branion, 1959, 1961; Motzok, 1963) as a means of evaluating the effect of dietary and other factors on fowl phosphatases, and the latter has been used to characterize isoenzymes of human tissue phosphatases (Moss, Campbell, Anagnostou-Kakaras & King, 1961).

Kinetic studies by these workers, by Zittle & Bingham (1960) and by Lazdunski & Ouellet (1961, 1962) have not established a satisfactory explanation for either relationship. Moreover, the kinetic data obtained by different workers present a confusing picture. For instance, Morton (1957) found that Lineweaver–Burk reciprocal plots were non-linear and his K_m values were therefore designated by the substrate concentration at half-optimum velocity. On the other hand, Motzok (1959) recorded linear reciprocal plots but found that K_m depended on the enzyme concentration.

In the present paper we have studied the kinetic behaviour of alkaline phosphatase by using a new substrate, 4-methylumbelliferyl phosphate monoester. The phosphate previously described by Leaback (1961) as one of a series of 4-methylumbelliferone conjugates was later found to be the P^1P^2-pyrophosphate diester (cf. Walker & King, 1950) and was not hydrolysed by serum phosphomonoesterases (J. W. Woollen, personal communication). 4-Methylumbelliferyl phosphate has a significant advantage over currently available substrates in that its useful concentration range extends to about 0·1 μM. Theoretically even lower concentrations could be employed, but non-enzymic hydrolysis imposed a practical limitation at this level.

The effects of pH, ionic strength and temperature on the hydrolysis of 4-methylumbelliferyl phosphate by an alkaline phosphatase from calf-intestinal mucosa are described below. A preliminary account of some of this work has appeared (Fernley & Walker, 1964).

MATERIALS AND METHODS

Buffers. Once-recrystallized tris or 2-amino-2-methylpropane-1,3-diol (ammediol) and HCl were used. Unless otherwise indicated, pH values refer to the assay temperature. They were measured with a Pye Dynacap pH-meter, with 0·05M-sodium borate solution, pH 9·27 at 15°, for reference.

Enzyme. A purified alkaline phosphatase (EC 3.1.3.1) from calf intestinal mucosa (lot AP–I 15436; C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) was

used. It had about 15% of the activity of the purest preparation so far obtained (Portmann, 1957). A solution (100 mμg./ml.) in 0·1% bovine plasma albumin (crystallized; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) at 2° decreased in activity by about one-third in 6 weeks.

4-Methylumbelliferyl phosphate monoester. It is important that all reagents should be dry. 4-Methylumbelliferone (10 g.) in pyridine (20 ml.) was added with stirring to a cooled mixture of phosphoryl chloride (8·5 g.) and pyridine (40 ml.) such that the temperature remained below 5°. After 30 min., the reaction product (4-methylumbelliferyl phosphoryl chloride) was decomposed by the addition of ice-cold water (100 ml.). The solution was adjusted to pH 7·5 with 10 N-NaOH and solvents were removed under reduced pressure (below 65°). The residue was triturated with acetone (30 ml.) and extracted with warm aq. 80% (v/v) methanol (125 ml.). Diluting the methanolic solution with acetone (300 ml.) gave 4-methylumbelliferyl phosphate monoester disodium salt (6 g.). This was recrystallized from aq. 70% (v/v) ethanol. Infrared-absorption maxima (KCl disk) occurred at 1675, 1610, 1425, 1290, 1115 (triple peak), 990 and 770 cm.$^{-1}$, as measured with a Perkin–Elmer model 237 grating spectrophotometer. The free acid was obtained by treating an aqueous solution of the disodium salt with Amberlite IR-100 (H^+ form) ion-exchange resin. The effluent was evaporated to dryness. The residue was dissolved in ethanol, and, on diluting with diethyl ether, 4-methylumbelliferyl phosphate monoester, m.p. 214–216° (uncorr.), crystallized (Found C, 46·5; H, 3·8; P, 12·1. $C_{10}H_9O_6P$ requires C, 46·9; H, 3·5; P, 12·1%). The ultraviolet absorption showed $\lambda_{max.}$ (in 0·5 M-K_2HPO_4–KOH buffer, pH 10·4) 320 mμ (log ε, 4·12). The titration curve indicated two acid groups, pK_1 about 1·7 and pK_2 5·6 at 20°.

Assay procedure. All mixtures included 0·01% of albumin. This helped to stabilize the enzyme at pH 8 and below. Buffer, water, albumin, enzyme and substrate (total volume 1·0 ml.) were incubated together in 12 ml. conical centrifuge tubes, and reaction was stopped by adding 1·0 ml. of M-K_2HPO_4–KOH buffer, pH 10·4. Controls were included to allow for non-enzymic hydrolysis of the substrate. Fluorescence was measured with a Locarte Mk. 4 fluorimeter, as described by Leaback & Walker (1961). Readings against a quinine standard were constant for at least 5 hr. Details of the various assays are as follows.

(*a*) Time-course of reaction. Buffer (0·05 M-ammediol–HCl, pH 7·9 or 9·6), enzyme and substrate were incubated at 37° for up to 2 hr. In preincubation experiments, buffer and enzyme were preincubated at 37° for up to 2 hr. and subsequently incubated with substrate for 30 min.

(*b*) All other experiments. Buffer and enzyme were brought to the assay temperature and reaction was started by adding substrate. Incubations were for 30 min.

RESULTS

The time-course of reaction between alkaline phosphatase and 4-methylumbelliferyl phosphate in 0·05 M-ammediol–hydrochloric acid buffers is shown

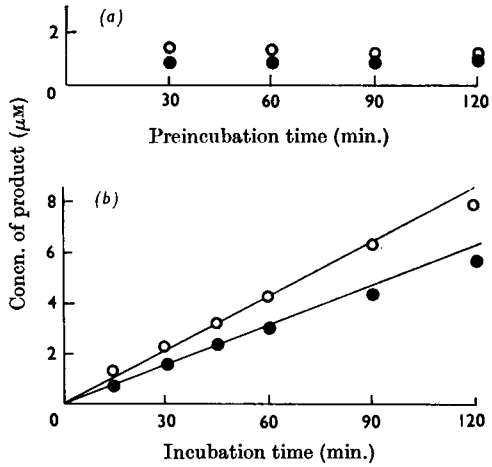


Fig. 1. Time-course of reaction (*b*) and preincubation effects (*a*) at 37°. ○, 0·05 M-Ammediol–HCl buffer, pH 7·9, 0·1 mM-substrate and 5 mμg. of enzyme; ●, 0·05 M-ammediol–HCl buffer, pH 9·8, 0·1 mM-substrate and 1 mμg. of enzyme. Other details are given in the Materials and Methods section.

Table 1. *Effect of* pH *on the hydrolysis of 4-methylumbelliferyl phosphate by alkaline phosphatase at* 30° *and I* 0·02

Ammediol–HCl buffers of the stated pH were used. Velocities are given as final concentrations of 4-methylumbelliferone (μM) produced enzymically in the reaction mixture (1·0 ml.) after 30 min. incubation. Other details are given in the Materials and Methods section.

pH of assay	Amount of enzyme (mμg.)	Initial concn. of substrate (μM)	Velocity ... x	$2x$	$4x$	$6x$	$8x$	$10x$	$15x$	$20x$	Value of x
8·05	0·5		0·041	0·065	0·091	0·102	0·117	0·126	0·138	0·145	0·5
8·32	0·25		0·021	0·034	0·053	0·064	0·074	0·087	0·091	0·104	0·5
8·63	0·5		0·099	0·158	0·242	0·283	0·318	0·348	0·389	0·405	1·0
8·92	0·5		0·148	0·252	0·377	0·455	0·510	0·542	0·607	0·631	2·0
9·22	1·0		0·503	0·843	1·28	1·54	1·71	1·88	2·01	2·09	5·0
9·52	1·0		0·58	1·03	1·66	2·07	2·27	2·59	2·73	3·02	10
9·83	2·0		1·44	2·41	4·08	5·12	5·96	6·75	7·37	7·69	25

Table 2. *Effect of* pH *on the hydrolysis of* 4-*methylumbelliferyl phosphate by alkaline phosphatase at* 30° *and I* 0·20

Details are as given in Table 1 and the Materials and Methods section.

pH of assay	Amount of enzyme (mμg.)	Initial concn. of substrate (μM) ...	Velocity x	$2x$	$3x$	$5x$	$7{\cdot}5x$	$10x$	Value of x
7·55	0·5		0·130	0·198	0·217	0·252	0·283	0·307	1·0
7·91	0·5		0·153	0·185	0·240	0·300	0·332	0·360	1·0
8·36	0·5		0·190	0·298	0·348	0·415	0·526	0·526	2·0
8·61	0·5		0·317	0·480	0·576	0·681	0·765	0·818	5·0
8·87	1·0		0·776	1·21	1·46	1·66	1·84	2·10	10
9·26	1·0		0·710	1·18	1·49	1·88	2·13	2·30	20

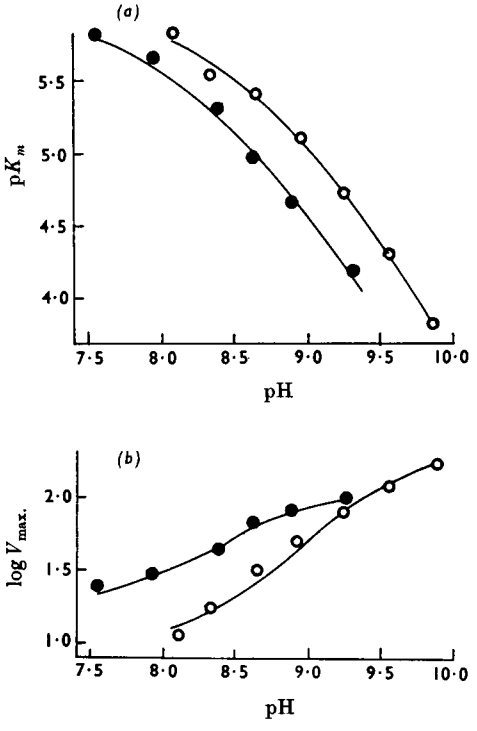


Fig. 2. Variation in $V_{max.}$ and K_m with pH at 30°. The experimental values are calculated from the data of Tables 1 and 2, and the curves derived from equations given in the text. $V_{max.}$ values are μmoles of substrate hydrolysed/min./mg. of enzyme. ○, *I* 0·02; ●, *I* 0·2.

in Fig. 1(*b*). Activity is linear for 60min. at both ends of the buffer range, and the usual incubation period of 30min. is therefore satisfactory for determining initial velocities. Preincubation effects (Fig. 1*a*) are slight and should not be significant under assay conditions where the maximum preincubation time was 20min. The rate of hydrolysis of 0·1mM-substrate in 0·05M-ammediol–hydrochloric acid buffer pH 9·6 at 37° was first-order with respect to enzyme concentration over a 20-fold range (0·1–2·0mμg. of enzyme). The addition of magnesium chloride (5mM) increased the activity of the enzyme by up to 100%. This activation varied with the concentration of substrate and magnesium chloride as described by Morton (1957). For this reason, Mg^{2+} was not added to the assay mixtures.

Effect of substrate concentration. Experiments in which the substrate concentration was varied at different pH values are set out in Tables 1 and 2. All plots of $1/v$ against $1/s$ are linear, in agreement with the findings of Zittle & Bingham (1960) and Lazdunski & Ouellet (1961) with phenyl phosphate and *p*-nitrophenyl phosphate respectively. Morton (1957), using phenyl phosphate in the presence of 0·01M-Mg^{2+}, did not obtain linear plots of $1/v$ against $1/s$, and it seems likely that this effect is attributable to the concentration of Mg^{2+} employed. The value of K_m at any given pH was found to be independent of the enzyme concentration [Motzok (1959) found K_m for glycerophosphate and chicken-plasma phosphatase to be inversely proportional to the enzyme concentration over a twofold range].

There was no inhibition by excess of substrate in the concentration ranges for determining K_m and $V_{max.}$ values, but at higher relative substrate concentrations ($s/K_m > 10$) inhibition was observed, especially at high pH.

Effect of pH. From the results given in Tables 1 and 2, variation in K_m and $V_{max.}$ with pH at constant ionic strength (0·02 and 0·2) can be evaluated. This is shown in Figs. 2(*a*) and 2(*b*). Values of K_m and $V_{max.}$ were obtained statistically by fitting a linear form of the Michaelis–Menten equation (Wilkinson, 1961).

The log $V_{max.}$ plots (Fig. 2*b*) indicate that a basic form of the enzyme–substrate complex is the most active one, and they also show that $V_{max.}$ values at

lower pH are greater than would be predicted on the basis of a single reactive ionic species. The pK_m plots (Fig. 2*a*) have non-integral slopes in the pH region of highest activity. Ionization of the substrate is complete in the pH range 8–10, so the pK_m plot must represent the behaviour of the enzyme. The non-integral slopes present a difficult problem in interpretation which is discussed below.

There is an empirical relationship between the K_m and $V_{max.}$ values of Fig. 2 such that in each case $V_{max.} = \beta/(1+\alpha/K_m)$, where α and β are constants (Fig. 3). This equation is of the same form as the Michaelis equation with $V_{max.}$ replacing v and K_m instead of s. Values of α and β given in Table 3 were obtained statistically by the procedure employed for determining K_m and $V_{max.}$. Empirical equations that relate the K_m and $V_{max.}$ values to these constants and to the H^+ concentration are of the form:

$$K_m = \frac{\alpha\left\{b+\frac{K_A}{[H^+]}\left(c+\frac{K_A d}{[H^+]}\right)\right\}}{1+\frac{K_A}{[H^+]}} \quad (1)$$

$$V_{max.} = \frac{\beta\left\{b+\frac{K_A}{[H^+]}\left(c+\frac{K_A d}{[H^+]}\right)\right\}}{1+b+\frac{K_A}{[H^+]}\left(1+c+\frac{K_A d}{[H^+]}\right)} \quad (2)$$

where b is a constant defining the flattening of the pK_m and log $V_{max.}$ curves at low pH, and c and d are constants that determine the slope at higher pH. K_A is an acid–base dissociation constant. The theoretical curves in Fig. 2 were calculated from eqns. (1) and (2) by using chosen values of the other constants given in Table 3.

The development of these equations, and a kinetic scheme that interprets β as a maximum $V_{max.}$ value and α as a pH-independent K_m term, are presented in the Discussion section.

Effect of ionic strength. As well as the measurements made at various pH values at two ionic strengths (Figs. 2*a* and 2*b*), K_m and $V_{max.}$ values

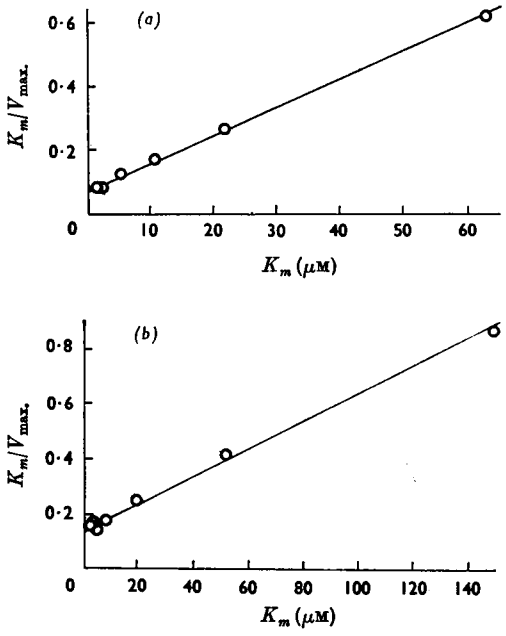


Fig. 3. Relationship between $V_{max.}$ and K_m, with varying pH, at 30° and I 0·02 (*b*) or I 0·2 (*a*). The experimental values are calculated from the data of Tables 1 and 2. The lines are described by the equation $K_m/V_{max.} = \alpha/\beta + K_m/\beta$; values for the constants α and β are given in Table 3.

Table 4. *Effect of ionic strength on K_m and $V_{max.}$ values at* pH 9·2 *and* 30°

Ammediol–HCl buffers of the stated ionic strength were used. K_m and $V_{max.}$ values were determined from velocity measurements at six different substrate concentrations as in Table 2. $V_{max.}$ values are μmoles of substrate hydrolysed/min./mg. of protein. Other details are given in the Materials and Methods section.

Ionic strength	pH	K_m (μM)	Coeff. of variation of K_m	$V_{max.}$	Coeff. of variation of $V_{max.}$
0·002	9·15	8·94	0·11	40·4	0·05
0·005	9·17	13·5	0·07	59·2	0·04
0·01	9·21	11·4	0·07	62·3	0·02
0·02	9·21	18·3	0·07	81·0	0·02
0·05	9·23	26·9	0·05	99·8	0·02
0·01	9·24	44·0	0·06	99·4	0·02
0·20	9·26	62·8	0·03	101·0	0·01

Table 3. *Values for the constants relating equations* (1) *and* (2) *with the theoretical curves of Fig.* 2

Ionic strength	α (μM)	Coeff. of variation of α	β (μmoles/mg./min.)	Coeff. of variation of β	pK_A	b	c	d
0·02	25·6	0·09	196·7	0·03	10·13	0·045	3·0	28
0·20	7·26	0·07	111·2	0·02	9·66	0·16	10	50

were determined at constant pH (9·2) over the range I 0·002–0·2. The results (Table 4) show that, at constant pH, $V_{max.}$ tends to approach a limiting value with increasing ionic strength, whereas K_m increases progressively. It is, however, difficult to compare K_m and $V_{max.}$ values obtained at the same pH but at different ionic strengths because, as Fig. 2 shows, a tenfold change in ionic strength causes the pH–activity curve to shift towards lower pH by 0·4–0·5 unit. We may conclude that increasing the ionic strength of the medium has at least three effects on alkaline phosphatase: (*a*) there is a decrease in catalytic activity, (*b*) there is an increase in affinity for substrate, which is masked by (*c*) an acid shift in the pH functions of K_m and $V_{max.}$. It is difficult to explain all these changes simply on the basis of decreased electrostatic interaction (Lazdunski & Ouellet, 1961; see the Discussion section), and it is possible that increased hydrophobic interactions may be important.

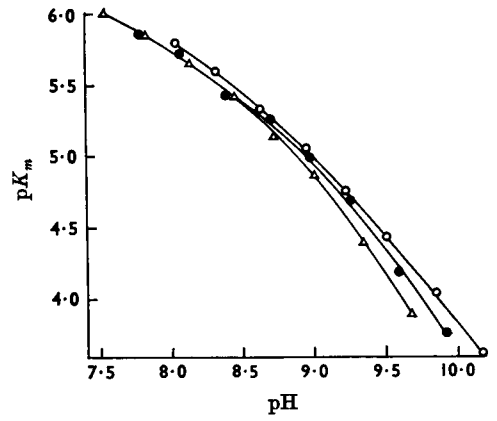


Fig. 4. Effect of pH and temperature on K_m. Values of pK_m (○, 20°; ●, 30°; △, 40°) for a 0·05 M-ammediol–HCl buffer system are calculated from sets of data similar to those given in Tables 1 and 2. The curves have no theoretical significance. pH values refer to the temperature of assay.

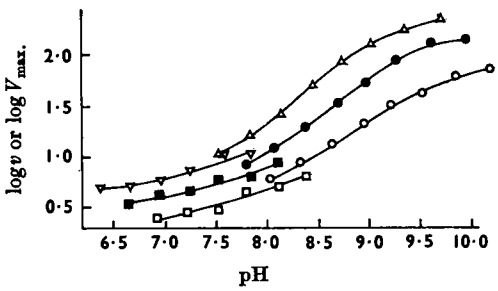


Fig. 5. Effect of pH and temperature on $V_{max.}$. Values of log $V_{max.}$ (○, 20°; ●, 30°; △, 40°) for a 0·05 M-ammediol–HCl buffer system are calculated from velocity measurements at high substrate concentration by using the K_m values given in Fig. 4. The additional values, log$v$ at high substrate concentration (□, 20°; ■, 30°; △, 40°), refer to a 0·05 M-tris–HCl buffer system. All velocities are expressed as μmoles of substrate hydrolysed/min./mg. of enzyme. pH values refer to the temperature of assay.

Effect of temperature. Values of pK_m and log V_{max} in the pH range 8–10 (0·05 M-ammediol–hydrochloric acid buffers) and temperature range 20–40° are given in Figs. 4 and 5. $V_{max.}$ values were estimated from activities at high substrate concentration by using the Michaelis equation and the K_m values given in Fig. 4. This procedure was adopted because the stock enzyme solution gradually lost activity during the course of the experiment. Additional results for a 0·05 M-tris–hydrochloric acid buffer system are included in Fig. 5. K_m values could not be determined because of the very high affinity for substrate in this pH region. Velocities are those observed with 10 μM-substrate and may be somewhat lower than the $V_{max.}$ values.

The results in the preceding section indicated that constant-molarity buffers should be used with caution for kinetic experiments since the ionic strength will vary tenfold over the useful pH range. In this experiment, however, we are interested in changes in K_m and $V_{max.}$ with temperature and not in comparing their absolute values at constant temperature (varying pH), so that it should be possible to obtain valid results.

From plots of $1/V_{max.}$ against $[H^+]$ or of $K_m/V_{max.}$ against K_m, it was deduced that the activity curve undergoes an acid shift along the pH axis of 0·46 unit between 20 and 40°. The corresponding heat of ionization is 9·6 kcal./mole, but this probably refers to a complex system rather than to a single ionizing group (see the Discussion section). The important point is that, to compare K_m and $V_{max.}$ at different temperatures, one must allow a fall of 0·23 pH unit/10° rise in temperature. The experimental values cannot be used directly and the following procedure was therefore adopted. A smooth curve was drawn through each set of values (see Figs. 4 and 5) and comparable points on each curve were selected. For instance, a point on the 20° log $V_{max.}$ curve at pH 9·50 was compared with the pH 9·27 point on the 30° curve and the pH 9·04 point on the 40° curve. Plots of pK_m against $1/T$ were linear throughout the pH range 8–10, but there was some doubt about the linearity of the plot of log $V_{max.}$ against $1/T$ at high pH. A separate experiment at pH 9·25 (30°, 0·02 I) in which $V_{max.}$ was determined every 3° over the range 20–40° established that the Arrhenius plot was linear.

The variation in ΔH (at 30°) with pH (at 30°) is shown in Fig. 6. For $V_{max.}$ this is a starred quantity,

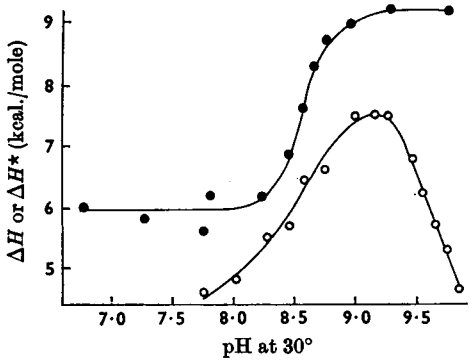


Fig. 6. Effect of pH on ΔH^* ($V_{max.}$) and ΔH (K_m). Thermodynamic constants for $V_{max.}$ (●) and K_m (○) are derived from the data of Figs. 4 and 5 by the procedure described in the text.

but for K_m the significance of ΔH will depend on the nature of K_m. Both values are maximum at pH 9·2, with ΔH^* ($V_{max.}$) about 9·2 kcal./mole and ΔH (K_m) about 7·5 kcal./mole.

DISCUSSION

Relationship between substrate concentration and optimum pH *or velocity.* If the concentration of 4-methylumbelliferyl phosphate is held constant and the pH is varied, the activity of the enzyme passes through a maximum. The height ($v_{opt.}$) and position ($pH_{opt.}$) of the maximum varies with the substrate concentration in the manner described by Ross *et al.* (1951), Motzok (1959) and Moss *et al.* (1961). We have found that the relationship between K_m and $V_{max.}$ (Fig. 3) gives rise to expressions that describe optimum pH and velocity in terms of the pH-independent constants α and β. Since

$$v = \frac{V_{max.}}{1+K_m/s} \quad \text{and} \quad V_{max.} = \frac{\beta}{1+\alpha/K_m}$$

it follows that

$$v = \frac{\beta}{(1+K_m/s)(1+\alpha/K_m)}$$

When $v = v_{opt.}$, $(\partial v/\mathrm{d}[H^+])_s = 0$. From Figs. 2(*a*) and 4 it is evident that $\mathrm{d}K_m/\mathrm{d}[H^+]$ is never zero; therefore, when $v = v_{opt.}$, $(\partial v/\mathrm{d}K_m)_s = 0$. It can readily be shown that under these conditions:

$$K_m = \sqrt{\alpha s}$$

$$v_{opt.} = \frac{V_{max.}}{1+\sqrt{\alpha/s}} = \frac{\beta}{(1+\sqrt{\alpha/s})^2}$$

A plot of $1/\sqrt{v_{opt.}}$ against $1/\sqrt{s}$ should therefore be linear with slope $\sqrt{\alpha/\beta}$ and intercept $\sqrt{1/\beta}$.

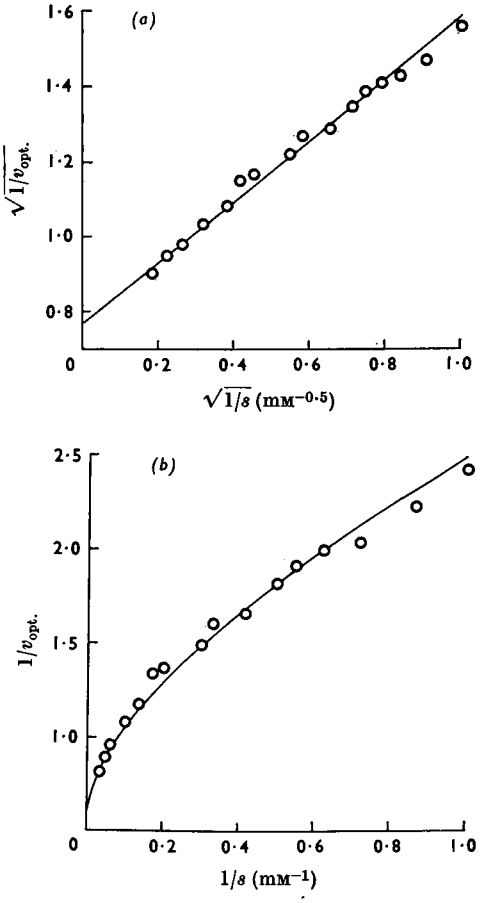


Fig. 7. Relationship between optimum velocity and substrate concentration with varying pH. The data are from Fig. 7 of Motzok (1959): (*b*) shows the original plot, but with a theoretical curve according to the equation:

$$1/v_{opt.} = 1/\beta + \alpha/\beta s + 2\sqrt{\alpha/\beta}\sqrt{s}$$

α (1·12 mM) and β (1·68) are obtained from the linear plot (*a*):

$$\sqrt{1/v_{opt.}} = \sqrt{1/\beta} + \sqrt{\alpha/\beta s}$$

Motzok (1959) interpreted a plot of $1/v_{opt.}$ against $1/s$ as showing two distinct slopes from which he obtained two 'K_m' values. If the above equations apply to this situation, the plot should be a continuous curve. In Fig. 7(*b*) we have replotted the data presented in Motzok's (1959) Fig. 7 and included a theoretical curve using constants obtained from the plot of $1/\sqrt{v_{opt.}}$ against $1/\sqrt{s}$ (Fig. 7*a*).

Further, since $K_m = \sqrt{\alpha s}$, it follows that, in the pH region where pK_m is linear with pH, a plot of $-\log s$ against $pH_{opt.}$ should also be linear but with twice the slope. Motzok & Branion (1959, 1961) and

Motzok (1963) give many examples of this plot, invariably linear, with slopes about -2.

Both the above types of result were obtained with different enzyme preparations, substrates and buffers; therefore we conclude that the relationship between K_m and $V_{max.}$ is generally valid.

Development of a kinetic scheme. The isolation by Engström (1961, 1962) of a phosphoryl-enzyme intermediate, previously implicated in the hydrolase and transferase activities of alkaline phosphatase (Morton, 1953), suggests that the following mechanism (Gutfreund & Sturtevant, 1956) is applicable, where ES denotes the enzyme–substrate complex, EX the phosphoryl-enzyme, $[E_t]$ the total enzyme concentration, and P_1 and P_2 are ROH and inorganic phosphate respectively:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow[P_1]{k_2} EX \xrightarrow[P_2]{k_3} E$$

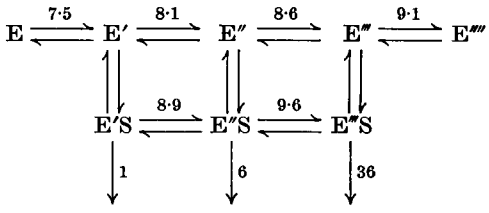
with

$$K_m = \frac{k_3(k_{-1}+k_2)}{k_{+1}(k_2+k_3)} \quad \text{and} \quad V_{max.} = \frac{[E_t]\,k_2\,k_3}{(k_2+k_3)}$$

Since K_m and $V_{max.}$ vary with pH, it is necessary to introduce additional equilibria. However, our results show that the pH-variation of K_m and $V_{max.}$ is not in accordance with theory (Dixon, 1953; Laidler, 1955). These features are not peculiar to this enzyme, substrate or buffer. Motzok (1959) recorded non-integral plots of pK_m against pH with chicken-plasma phosphatase, phenyl phosphate and veronal–carbonate buffers, as did Lazdunski & Ouellet (1961) with a calf intestinal-mucosal enzyme, *p*-nitrophenyl phosphate and tris– or ethanolamine–hydrochloric acid buffers. The latter workers also found anomalous $V_{max.}$ values at low pH and suggested that electrostatic effects were responsible. Since the enzyme activity at low pH was further enhanced by increasing the ionic strength of the buffer, it is unlikely that electrostatic effects are responsible for the high $V_{max.}$ values. There is no evidence to suggest that a second enzyme with a different pH optimum is present, and so we must conclude either that there is more than one reactive form of the enzyme, or that the pH-dependent equilibrium has a pH-independent component.

Interpretation of the results in Fig. 2 in terms of multiple ionic species requires five free enzyme and three reactive ES species. The steady-state treatment gives rise to a very complex equation that in general will not give Michaelis–Menten kinetics. In a case such as this, where Michaelis–Menten kinetics are observed, it is usual to interpret the results as though equilibrium kinetics applied. For instance, Scheme 1 is applicable to the results for *I* 0·02.

There are several objections to this interpretation. First, the proximity of the molecular dissociation



Scheme 1. The figures given are the pK values of the respective dissociation constants and the relative rate constants for $V_{max.}$.

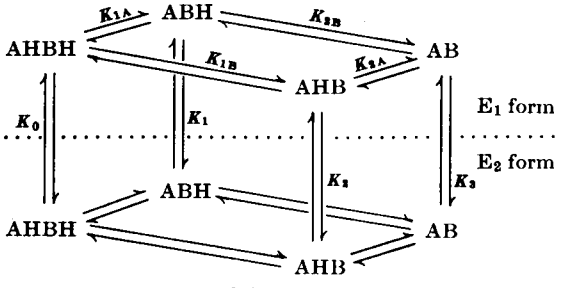
constants indicates that the group constants are virtually identical. Since all the groups are presumed to be at the active site, it seems unlikely that no interaction should be evident. Secondly, at higher ionic strength an analogous scheme with different rate constants must be formulated and it is difficult to reconcile this with purely electrostatic effects. Thirdly, this theory offers no explanation for the observed relationship between K_m and $V_{max.}$. We have therefore tried to develop a scheme that does offer a reasonable explanation for the K_m–$V_{max.}$ relationship. The general requirement appears to be that two forms of the enzyme E_1 and E_2 are related by a pH-dependent equilibrium such that $K_m = \alpha[E_2]/[E_1]$ and $V_{max.} = \beta[E_2]/([E_1]+[E_2])$.

In our view the most attractive interpretation is that E_1 and E_2 are different conformations of alkaline phosphatase; only E_1 can combine with substrate; only E_2 can react to give products. A pH-dependent conformational equilibrium of this type may be analysed by using the model described by Tanford (1961). The simplest scheme compatible with the observed pH effects requires that the change in conformation be associated with two ionizing groups changing their pK values. These groups need not be related to one another or to the active site.

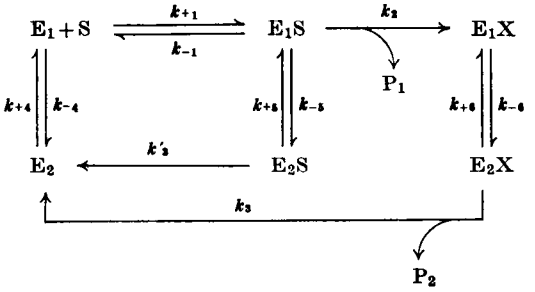
We have considered the situation where two basic groups A and B have normal pK values in the E_1 form (pK values similar to those exhibited by the same groups in analogous simple molecules) but are weaker bases in the E_2 form. Such a weakening could occur if the groups concerned were transferred to a more non-polar environment. Thus one might expect that the above process would be associated with a tightening of conformation and a 'squeezing out' of water so that the E_2 form would be more favourable for an un-ionized base.

In the system illustrated in Scheme 2, each ionic species is linked to the corresponding member in the second conformation by a pH-independent equilibrium:

$$\frac{[AHBH]_1}{[AHBH]_2} = K_0 \qquad \frac{(K_{1A})_2}{(K_{1A})_1} = \frac{K_1}{K_0} \text{ etc.}$$



Scheme 2.



Scheme 3.

Five of the 12 equilibrium constants are redundant and it is convenient to express the pH functions in terms of the normal E_1 dissociation constants. We then have:

$$\frac{[E_2]}{[E_1]} = \frac{K_0+\frac{K_{1A}}{[H^+]}\left(K_1+\frac{K_2K_{2B}}{K_{2A}}+\frac{K_3K_{2B}}{[H^+]}\right)}{1+\frac{K_{1A}}{[H^+]}\left(1+\frac{K_{2B}}{K_{2A}}+\frac{K_{2B}}{[H^+]}\right)} \quad (3)$$

$$\frac{[E_2]}{[E_1]+[E_2]} = \frac{K_0+\frac{K_{1A}}{[H^+]}\left(K_1+\frac{K_2K_{2B}}{K_{2A}}+\frac{K_3K_{2B}}{[H^+]}\right)}{1+K_0+\frac{K_{1A}}{[H^+]}\left\{1+K_1+\frac{(1+K_2)K_{2B}}{K_{2A}}+\frac{(1+K_3)K_{2B}}{[H^+]}\right\}} \quad (4)$$

The experimental plots of Fig. 2 can be fitted to eqns. (3) and (4), which simplify to eqns. (1) and (2) if the ionization of AH in the E_1 form is independent of BH($K_{1A} = K_{2A}$). Thus a possible solution for the *I* 0·021 results would be $pK_{1A} = pK_{2A} = 10{\cdot}13$, $pK_{2B} = 11{\cdot}68$, $K_0 = 0{\cdot}045$, $K_1 + (K_2/36) = 3{\cdot}0$ and $K_3 = 1000$. In the pH range considered both K_{2B}/K_{1A} and $K_{2B}/[H^+]$ are much less than 1, and eqn. (3) simplifies to:

$$\frac{[E_2]}{[E_1]} = \frac{K_0+\frac{K_{1A}}{[H^+]}\left(K_1+\frac{K_2K_{2B}}{K_{2A}}+\frac{K_3K_{2B}}{[H^+]}\right)}{1+\frac{K_{1A}}{[H^+]}}$$

This is of the same form as eqn. (1) with $K_{1A} = K_{2A} = K_A$, $K_0 = b$, $K_1 + (K_2K_{2B}/K_{2A}) = c$ and $K_3K_{2B}/K_{1A} = d$. Though it is important to show that this particular model is capable of explaining the experimental results, we consider that undue emphasis should not be placed on the detailed solutions because almost certainly other factors, e.g. electrostatic effects, will modify the pH behaviour of alkaline phosphatase and we cannot at present correct for these.

The kinetic scheme to include a pH-dependent conformational equilibrium is shown in Scheme 3, with:

$$K_m = \frac{(k_{+4}+k_{-4})\left\{k_{-1}+k_2+k_{+5}-\frac{k_{+5}k_{-5}}{(k'_3+k_{+5})}\right\}}{k_{+1}\left\{Ck_{+4}+k_2+\frac{k'_3k_{-5}}{(k'_3+k_{+5})}\right\}}$$

$$V_{max.} = \frac{[E_t]k_2k_{+4}}{Ck_{+4}+k_2+\frac{k'_3k_{-5}}{(k'_3+k_{+5})}}$$

and

$$C = 1+\frac{k_2}{k_3}+\frac{k_{-5}}{(k'_3+k_{+5})}+\frac{k_2(k_3+k_{+6})}{k_3k_{-6}}$$

This complex steady-state equation is completely general and reduces to the form $V_{max.} = \beta/(1+\alpha/K_m)$ under certain special conditions. For instance, if one assumes that the conformational changes are slow compared with the other rates, the only kinetically important rate constants will be k_{+1}, k_{-1}, k_2, k_{+4}, k_{-4} and k_{-6}, and, provided that $k_{-1} \ll k_2$:

$$K_m = \frac{(k_{+4}+k_{-4})k_2}{k_{+1}k_{+4}\left(1+\frac{k_2}{k_3}+\frac{k_2}{k_{-6}}+\frac{k_2}{k_{+4}}\right)} = \frac{k_{-6}(k_{+4}+k_{-4})}{k_{+1}(k_{+4}+k_{-6})}$$

and

$$V_{max.} = \frac{[E_t]k_{+4}}{1+\frac{k_{+4}}{k_{-6}}};\quad \alpha = \frac{k_{+4}(k_{+4}+k_{-4})}{k_{+1}(k_{+4}+k_{-6})};\quad \beta = [E_t]k_{+4}$$

For α and β to be constant in the pH range 8–10, k_{+4} must be constant and $k_{-4} = k_{-6}$. Hence:

$$K_m = \frac{k_{-4}}{k_{+1}},\quad V_{max.} = \frac{[E_t]k_{+4}}{1+\frac{k_{+4}}{k_{-4}}} \text{ and } \alpha = \frac{k_{+4}}{k_{+1}}$$

Alternatively, if one assumes that E_1 and E_2, E_1X and E_2X are at equilibrium and have the same

equilibrium constant, and also that $k_2 \gg k_{-1}$ and k_{-5}, the steady-state equation gives:

$$K_m = \frac{k_2 k_3 k_{-4}}{k_{+1} k_{+4}(k_2 + k_3)}$$

and

$$V_{max.} = \frac{[E_t] k_2 k_3}{(k_2 + k_3)\left(1 + \frac{k_{+4}}{k_{-4}}\right)};$$

$$\alpha = \frac{k_2 k_3}{k_{+1}(k_2 + k_3)}; \quad \beta = \frac{[E_t] k_2 k_3}{(k_2 + k_3)}$$

It is implied in both schemes that all ionic species of E_1 are equally reactive towards the substrate, and in the latter scheme it is also implied that all ionic species of E_2X are equally reactive. This would be possible if the groups involved in the conformational change were not at the active site.

Some thermodynamic considerations. With regard to the effect of pH on $\Delta H^\star$ ($V_{max.}$), we can interpret the difference between $\Delta H^\star$ at low and high pH (Fig. 6) as the heat of conversion of E_1 into E_2. In the case where the conformational changes are rate-limiting it is possible to evaluate all the thermodynamic quantities. On the basis that the enzyme preparation employed was 15% pure and that the mol.wt. is 100000 (Engström, 1961), k_{+4} should be about 2×10^3sec.$^{-1}$. A set of values for pH 9·3, 30° and 0·02I is given in Table 5.

Owing to the opposing effect of the proposed conformational equilibrium on the heat of ionization, the true heat will be 3·2kcal./mole greater than the apparent heat. At about 13kcal./mole, therefore, the groups involved are likely to be lysine or arginine residues. The negative entropy change indicates a tightened conformation for E_2, so that a basic group, if affected, should be weaker in the E_2 form.

The thermodynamics of binding can only be evaluated if it is assumed that $k_{-1} \ll k_2$. We have already made use of this proviso, and with $k_{+4} = 2 \times 10^3$sec.$^{-1}$ it follows that at pH 9·3 (30°, I 0·02), $\Delta G^\star$ (k_{+1}) = 5·4kcal./mole, $\Delta H^\star$ (k_{+1}) = 16·7 kcal./mole and $\Delta S^\star$ (k_{+1}) = 39 entropy units/mole.

Mechanism of alkaline-phosphatase action. A detailed analysis of the kinetic data has led us to conclude that the effects of pH, ionic strength and temperature are best explained in terms of a pH-dependent conformational equilibrium. The kinetic scheme that was developed requires that the enzyme combines with substrate in one conformation (E_1) but reacts to give inorganic phosphate in another conformation (E_2). Jencks (1963) has pointed out that covalent catalysis requires unusual properties of a catalyst in that it must be both a good acceptor and an efficient donor. Our scheme conforms with this requirement since it implies that the enzyme can assemble two active sites, one for phosphorylation of the enzyme and the other for the transfer of phosphate to a suitable acceptor.

Table 5. *Thermodynamic constants for the proposed conformational equilibrium*

The values refer to 30°, pH 9·3 and I 0·02, and are based on two assumptions: that the conformational changes are rate-limiting and that the velocity constant $k_4 = 2 \times 10^3$sec.$^{-1}$.

(First-order) rate constant	$\Delta G^\star$ (kcal./mole)	$\Delta H^\star$ (kcal./mole)	$\Delta S^\star$ (entropy units/mole)
k_{+4}	13·2	9·2	−13·2
k_{-4}	13·2	6·0	−23·7

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