THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 245, No. 15, Issue of August 10, pp. 3956-3963, 1970
Printed in U.S.A.

The Effect of pH, Temperature, and Organic Solvents on the Kinetic Parameters of *Escherichia coli* Alkaline Phosphatase*

(Received for publication, February 4, 1970)

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SUMMARY

A study of the effect of pH on kinetic parameters of *Escherichia coli* alkaline phosphatase, in presence and in absence of organic solvents, has shown that a cationic acid group with a pK of 7.4 at 25.5° is implicated in catalysis and that a neutral acid group with a pK of 9.2 at 25.5° is involved in enzyme-substrate binding. From the change in pK with temperature, values of 6,500 and 11,000 cal per mole, respectively, have been calculated for the heats of ionization of these two groups. These data are consistent with the proposal that the imidazolium group of a histidine residue participates in catalysis, and that a water molecule coordinated to the zinc atom functions in enzyme-substrate binding.

Construction of a reaction mechanism for an enzyme requires a knowledge of its amino acid or other residues involved in the catalytic process. Such information can be obtained either by organic chemical or kinetic methods. In the first procedure, a residue can be identified as important for enzyme action, if its chemical modification results in a corresponding loss in enzyme activity or if it forms a stable covalent link with a substrate moiety. In the second method, a detailed kinetic study of the enzyme reaction can be carried out under a variety of conditions which affect the ionization of one or more residues participating in the reaction. From these studies it is possible to gather information regarding the charge types at the active center and their pK values. This knowledge enables the investigator to make reasonable guesses regarding the amino acid or other residues of the enzyme participating in the formation of enzyme substrate complex or in catalysis. In spite of its obvious limitations, the kinetic method has been used to great advantage in understanding the mechanism of action of a few enzymes, notable among which is ribonuclease (1–3). As part of our efforts to understand the function of *Escherichia coli* alkaline phosphatase, we initiated studies on the variation of Michaelis parameters of the enzyme under different conditions of pH, temperature, and solvent composition. The object was to tentatively identify certain residues of the enzyme as being important for its function. The present communication summarizes these studies. Similar studies have been reported earlier for *E. coli* alkaline phosphatase by Lazdunski and Lazdunski (4, 5). Recently, Gottesman, Simpson, and Vallee (6) described kinetic studies on *E. coli* alkaline phosphatase in which zinc was replaced by cobalt.

E. coli alkaline phosphatase catalyzes a multistep reaction in which the hydrolysis of phosphate esters involves a phosphoryl-enzyme as an intermediate (7–9). The pathway of this reaction, originally proposed by Schwartz (10), can be represented as follows:

$$E + \mathrm{ROP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E - \mathrm{ROP} \xrightarrow{k_2} \mathrm{ROH} \qquad (1)$$

$$EP \xrightarrow{k_3} E + P$$

where E, enzyme; ROP, phosphate ester; E-ROP, enzyme-substrate complex; EP, phosphoryl-enzyme; ROH, leaving group; P, orthophosphate; k_1, rate constant for the association of enzyme and substrate; k_{-1}, rate constant for the dissociation of enzyme-substrate complex into free enzyme and substrate; k_2, rate constant for the phosphorylation of enzyme; k_3, rate constant for the dephosphorylation of phosphoryl-enzyme.

The experiments of Aldridge, Barman, and Gutfreund (11) and Fernley and Walker (12) show that around and above pH 7.0, the rate-determining step in this multistep reaction is the phosphorylation of the enzyme. However, some recent evidence (13) indicates that a substrate-dependent conformational change which follows the formation of enzyme-substrate complex and precedes the formation of phosphoryl-enzyme could be rate-determining in this pH range. Kinetic studies reported in this paper are based on rate measurements carried out in the pH range in which for the most part there is only one rate-controlling step. Hence, any conclusions drawn therefrom have relevance only to this step. Its exact nature is not of any consequence for the present work.

* Preliminary reports describing some of these studies were presented at Fourth Annual Symposium in Biophysics, Varanasi, India, November 1969, Abstract 27 and at Thirty Eighth Annual Meeting of the Society of Biological Chemists (India), Bombay, India, December 1969.

MATERIALS AND METHODS

Enzyme—The enzyme was obtained from *E. coli* strain $C_{97}F$ which was constitutive for alkaline phosphatase. The enzyme was routinely obtained by the following procedure essentially based on that of Malamy and Horecker (14). A culture of *E. coli* $C_{97}F$ grown overnight in Lederberg's broth was centrifuged and washed with 0.03 M Tris buffer, pH 9.0. The cells were resuspended in 0.03 M Tris buffer, pH 9.0, which was 0.5 M with respect to sucrose. This suspension had a cell titer of approximately 2×10^{11} cells per ml. To 10 ml of the suspension kept in an ice bath, 1 ml of 0.5% lysozyme solution was added and the mixture was stirred well for 1 min followed by the addition of 0.5 ml of 0.1 M EDTA to a final concentration of 0.005 M. The suspension was kept for 15 min in an ice bath with occasional mixing. Then, 0.5 ml of 1 M magnesium sulfate was added and the mixture was kept for 20 min in ice followed by centrifugation for 15 min at 10,000 rpm. The supernatant was heated at 65° for 10 min and centrifuged. The supernatant was dialyzed overnight against 50 volumes of 0.01 M Tris buffer, pH 7.4, containing 0.001 M magnesium sulfate and 0.005 M sodium chloride. The dialysate was treated with DNase and RNase at 37° for 30 min. It was then dialyzed for 2 hours against 50 volumes of the same buffer followed by another similar dialysis.

The dialysate was subjected to diethylaminoethylcellulose chromatography. The enzyme was eluted with a gradient of 0.005 M to 0.2 M sodium chloride in 0.01 M Tris buffer, pH 7.4, containing 0.001 M magnesium sulfate. The tubes of constant specific activity were pooled and dialyzed exhaustively against 0.01 M ammonium bicarbonate solution. The dialysate was lyophilized and stored in the deep freeze at −20°. The enzyme was homogeneous by ultracentrifugal analysis and had a specific activity of about 45 to 50 units per mg of protein (a unit being defined as the amount of enzyme which can convert 1 μmole of substrate, *p*-nitrophenyl phosphate, into product in 1 min at 30° under the conditions of assay).

Protein Concentration—Protein was measured spectrophotometrically at 278 mμ; 1 mg per ml of the purified protein gives an absorbance reading of 0.72 at 278 mμ in a cell with a 1-cm path length (15).

Materials—Disodium *p*-nitrophenyl phosphate tetrahydrate was obtained from Sigma. Maleic acid was obtained from Riedel De Haen AG, Seelze-Hanover, Germany. Sodium barbital and sodium carbonate were obtained from E. Merck AG, Darmstadt, Germany. AnalaR sodium bicarbonate was from British Drug Houses, Ltd., Poole, England. Phenol-*p*-sulfonate, ethylenediamine hydrochloride, and *N*-ethylmorpholine were AnalaR grade. Sodium hydroxide solutions of the required dilution were prepared by titration against standard succinic acid solutions. Concentrated hydrochloric acid of specific gravity 1.18, AnalaR grade, and 1,4-dioxane, AnalaR grade, were from British Drug Houses, Ltd. AR grade formamide was from Koch Light Laboratories, Colnbrook, England.

Buffers—All buffer solutions had an ionic strength of 0.27, the ionic strength being adjusted by the addition of sodium chloride. For kinetic studies other than those carried out in presence of organic solvents, the buffers employed were maleate, sodium barbital, and sodium bicarbonate-carbonate. All pH measurements were made at the temperature of the reaction with a Radiometer pH meter type 22 or type 4.

Measurement of Reaction Rates—All kinetic studies detailed here were performed using *p*-nitrophenyl phosphate as the substrate. Its rate of hydrolysis by alkaline phosphatase was calculated from the extinction change associated with release of the product, *p*-nitrophenol. Spectrophotometric records of *p*-nitrophenyl phosphate hydrolysis were made at 410 mμ. The *p*-nitrophenolate anion has an extinction coefficient of 1.91×10^4 at 410 mμ. The velocity is expressed as micromoles of *p*-nitrophenol formed per min per ml of reaction mixture under the conditions of the experiment. Generally, enzyme concentrations of 0.2 to 0.5 μg per ml were used. The concentration was varied appropriately for the various kinetic determinations described.

The assay medium (final volume, 2.4 ml) contained *p*-nitrophenyl phosphate of suitable concentration in an appropriate buffer solution. The reaction was initiated by the addition of enzyme after dilution with appropriate buffers.

The rates of hydrolysis were followed in a Carl-Zeiss spectrophotometer equipped with a Honeywell recording device and a temperature-controlled cell compartment using 1-cm glass or silica cuvettes. Measurements were made at higher temperature by circulating water by means of a Haake circulator fitted with an ultrathermostat. Measurements of K_m values and maximum velocity were made at temperatures in the range 24–42°. The reversibility of the pH effect on rate was tested at each pH by readjustment of the pH of alkaline phosphatase to 7.90 and reassaying the enzyme.

Measurement of K_m Values—For the determination of K_m value at each pH, eight different substrate concentrations were normally used ranging from 1×10^{-3} M to 5×10^{-6} M. K_m values were calculated from Lineweaver-Burk plots (16).

Measurement of Maximum Velocity—The effect of the concentration and type of buffer ions on reaction rates was eliminated by measuring velocities at any particular pH at varying buffer ion concentrations keeping the ionic strength constant. The velocities were extrapolated to zero buffer ion concentration and the extrapolated value was taken as the correct velocity. For measurements of maximum velocity, the concentration of substrate used was 2×10^{-3} M which was at least 25-fold in excess of the K_m at all pH values. The values of maximum velocity thus obtained agreed with those calculated from Lineweaver-Burk plots.

Kinetic Studies in Organic Solvent-Water Mixtures—The K_m and maximum velocity values for the enzyme in formamide-water or dioxane-water mixtures were obtained in the same way as described above. The measurements were made in two series of buffers: (*a*) cationic acid buffers and (*b*) neutral acid buffers. The cationic acid buffers employed were ethylenediamine hydrochloride and *N*-ethylmorpholine hydrochloride. The neutral acid buffers used were maleate and phenol-*p*-sulfonate. pH measurements of buffers in organic solvent-water mixtures were made using the procedure of Bacarella *et al.* (17).

The substrate was prepared in the buffer containing dioxane or formamide such that in the final reaction mixture, the substrate concentration was of the desired value, the ionic strength was 0.27, and the concentration of solvent was 25% (v/v).

RESULTS

Dependence of V_{max} on pH—Dependence of V_{max} on pH using *p*-nitrophenyl phosphate as substrate was analyzed by using the method of Dixon and Webb (18). According to their theoretical treatment, a plot of log V_{max} against pH gives a graph

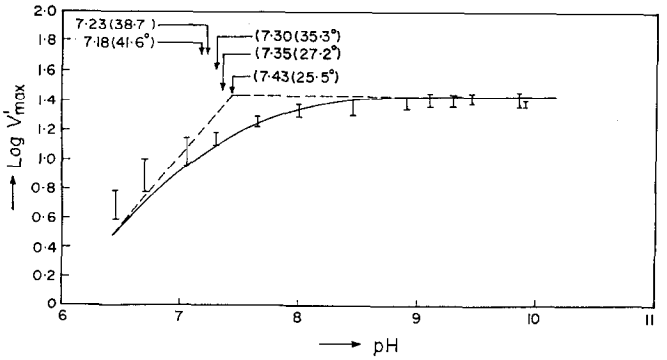

FIG. 1. Dixon plot of log V_{max} *versus* pH. Buffers employed were: pH 6.45 to 6.70, maleate; pH 7.05 to 8.45, sodium barbital; pH 8.90 to 9.85, sodium bicarbonate-carbonate. The *solid line* represents theoretically calculated values of log V_{max} at 25.5° (see text). The experimental points show the range of variation of five independent experiments at the same temperature. The *point of intersection of dotted lines* indicates $pK_{b,ES}$ value at this temperature. The $pK_{b,ES}$ values determined at other temperatures (shown in parentheses) are indicated by *arrows*. $V'_{max} = V_{max} \times 800$.

consisting of a number of straight line portions interconnected by short curved parts. The straight line segments have integrated slopes, *i.e.* 0 or 1-unit or 2-unit slopes, positive or negative. The segments change in slope in the pH region where the enzyme-substrate complex dissociates protons. Each bend in this graph indicates the pK of an ionizing group in the enzyme-substrate complex and the straight line portions when produced intersect at a pH corresponding to the pK values of these ionizing groups. The straight line portions intersect at a point 0.3-unit vertical distance from the experimentally determined graph. Analysis of such plots can thus tell about the ionizations which occur in the enzyme-substrate complex. On the basis of these ionizations, deductions can be made regarding the groups on the enzyme which have a determining effect on its activity and are thus a part of the catalytic site. Fig. 1 represents such an analysis. Points shown in this figure were experimentally determined. On the basis of these points, linear segments with +1 and 0 slopes have been constructed. These are shown as *dotted lines*. The pK value indicated by the point of intersection of these segments gives the ionization constant of a group in the enzyme-substrate complex. This ionization constant was employed to calculate a theoretical curve from the following equation

$$\log V_{max} = \log k_2E - \log\left(1 + \frac{(H^+)}{K_{b,ES}} + \frac{K_{a,ES}}{H^+}\right) \quad (2)$$

where k_2 is the catalytic rate constant and $K_{b,ES}$ and $K_{a,ES}$ are the ionization constants of groups on enzyme-substrate complex. The term log k_2E which is a constant independent of pH was evaluated from values of V_{max} at several hydrogen ion concentrations. Since the experimental points do not indicate any ionization corresponding to $pK_{a,ES}$ of enzyme-substrate complex, Equation 2 can be rewritten as

$$\log V_{max} = \log k_2E - \log\left(1 + \frac{(H^+)}{K_{b,ES}}\right) \quad (3)$$

The theoretical curve based on Equation 3 is shown as a *solid line* in Fig. 1. The visual fit between the theoretical curve and the experimental points is quite satisfactory above pH 7.0.

TABLE I

Ionization constants and heats of ionization for alkaline phosphatase at 0.27 ionic strength

	Temperature	$pK_{b,E}$	$pK_{b,ES}$	$pK_{a,E}$
Free enzyme[a]	25.5	7.30		9.18
	29.8			9.08
	33.0			8.93
	36.8	7.20		8.88
	39.2	7.10		8.83
Enzyme-substrate complex[b]	25.5		7.43	
	27.2		7.35	
	31.0		7.38	
	35.3		7.30	
	39.0		7.23	
	41.6		7.18	
Heats of ionization (cal)		6,200	6,500	11,000

[a] From log $(V_{max})/(K_m)$ and pK_m *versus* pH plots.
[b] From log V_{max} *versus* pH plots.

Below this pH however, the experimental points deviate from the theoretical curve. Such a deviation has also been observed by Gottesman *et al.* (6). The reason for this deviation could be the change in the rate-limiting step that occurs below pH 7.0 (12). At some stage between pH 6.0 and pH 7.0, there is a change in the rate-determining step from dephosphorylation of the enzyme at lower pH to its phosphorylation at higher pH. Thus an ionization in the enzyme-substrate complex that governs its activity above pH 7.0 will obviously be without effect at pH values where the rate-controlling step is the dephosphorylation of the enzyme.

Since the log V_{max} *versus* pH curve shows only one inflexion corresponding to $pK_{b,ES}$ of 7.43 at 25.5°, it is clear that only the group represented by this pK is the one which ionizes in the enzyme-substrate complex and which controls the catalytic activity of the enzyme. This group performs its catalytic function in the basic form.

Fig. 1 also shows the effect of alteration of temperature on the $pK_{b,ES}$ value of the enzyme. This pK decreases with increasing temperature. Using this data and the van't Hoff relationship

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2} \quad (4)$$

the heat of ionization of the group represented by this pK was shown to be about 6500 cal (Table I).

Similar results have been obtained by Lazdunski and Lazdunski (4) and by Gottesman *et al.* (6) for native and cobalt alkaline phosphatase, respectively.

Dependence of Velocity at Low Substrate Concentration on pH—Just as measurements of reaction rates at high substrate concentrations yield ionization constants for groups on the enzyme-substrate complex, measurements at very low substrate concentrations can be used to obtain information regarding the ionization constants of groups on the free enzyme and the substrate. Since at low substrate concentration, reaction velocity is given by

$$v = \frac{V_{max}}{K_m} \times S$$

where S is the concentration of the substrate, a plot of log $(V_{max})/(K_m)$ *versus* pH will give the pK values of the free enzyme and the free substrate (18). Such a plot has been described in Fig. 2. The points shown are based on the experimental determination of V_{max} and K_m at different pH values. With these points, linear segments with +1, 0, and −1 slopes have been drawn and are shown as *dotted lines*. The points of intersection of these linear segments give the pK values of ionizing groups on the free enzyme or the free substrate. Since the substrate *p*-nitrophenyl phosphate has a pK of 5.70 (19), it does not ionize in the pH range under investigation. These pK values ($pK_{a,E}$ and $pK_{b,E}$), therefore, give the ionization constants of the free enzyme only. These were employed to calculate a theoretical curve from the following equation

$$\log\left(\frac{V_{max}}{K_m}\right) = \log\left(\frac{k_2 E}{\bar{K}_m}\right) - \log\left(1 + \frac{(H^+)}{K_{b,E}} + \frac{K_{a,E}}{(H^+)}\right) \quad (5)$$

The term log $(k_2E)/(\bar{K}_m)$ which is a constant independent of pH was obtained from the values of $(V_{max})/(K_m)$ at several hydrogen ion concentrations. $K_{a,E}$ and $K_{b,E}$ are the ionization constants of the free enzyme and were obtained as shown above. The theoretical curve based on Equation 5 is shown as a *solid line* in Fig. 2. The visual fit between the experimental points and the theoretical curve is satisfactory. This indicates that the $pK_{a,E}$ and $pK_{b,E}$ values are correctly calculated.

The $pK_{a,E}$ value of 9.18 (25.5°) and the $pK_{b,E}$ value of 7.30 (25.5°), thus represent ionizable groups on the free enzyme which are involved in enzymatic activity. The $pK_{b,E}$ value of 7.30 is close to the $pK_{b,ES}$ value of 7.43 determined for the enzyme-substrate complex at the same temperature (Fig. 1). The ionization of the group represented by this pK is, therefore, unaffected by the combination of the enzyme with the substrate. This indicates that the group in question is merely concerned with the mechanism of catalysis and is not involved in binding with the substrate. Since from Fig. 1 this appears to be the only group which participates in catalysis, it follows that the other group represented by the $pK_{a,E}$ value of 9.18 (25.5°) in its acidic form functions in enzyme-substrate binding.

Fig. 2 also shows the effect of temperature on the $pK_{b,E}$ and $pK_{a,E}$ values of the enzyme. Using this data, the heats of ionization of the groups represented by these pK values were calculated to be 6,200 and 11,000 cal, respectively (Table I).

It is necessary to briefly mention here that Lazdunski and Lazdunski (4) have reported a $pK_{a,E}$ value of 8.60 which is considerably lower than the value of 9.18 observed by us. They also obtained a relatively high value of 17,500 cal for the heat of ionization of the group corresponding to this pK. Even considering that the conditions of their experiment were rather different from ours, it is not possible to explain this discrepancy. Gottesman *et al.* (6) have, however, reported a $pK_{a,E}$ value of 9.6 and a heat of ionization of 14,000 cal for this group in cobalt alkaline phosphatase.

Dependence of K_m on pH—The respective role of these two ionizable groups corresponding to pK values of 9.18 and 7.43 in the activity of the enzyme was further confirmed by an analysis of pH dependence of K_m using the method of Dixon (20). In this case a plot of pK_m ($-\log_{10} K_m$) against pH yields a curve that has properties similar to those of a V_{max} *versus* pH curve. Each bend in this curve indicates the pK of an ionizing group in either the free enzyme, the free substrate, or the enzyme-substrate complex. Bends in the curve concave to the pH axis correspond to the ionization of a group in the free enzyme or the free substrate. Bends convex to the pH axis are produced by the ionization of a group in the enzyme-substrate complex. This type of analysis is presented in Fig. 3. The points shown in this figure were experimentally obtained. Based on these points and using the same procedure as described for Figs. 1 and 2, a pK value of 9.18 at 25.5° is calculated for a bend concave to the pH axis. Since as pointed out earlier, the substrate does not ionize in this pH range, it is clear, that the pK value of 9.18 corresponds to the ionization of a group on the free enzyme, *i.e.* its $pK_{a,E}$. This $pK_{a,E}$ value was employed to calculate a theoretical curve for the relationship between the pK_m and the pH

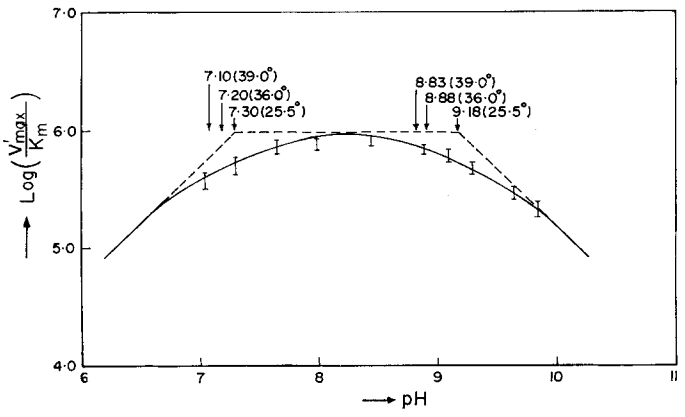

FIG. 2. Dixon plot of log $(V_{max})/(K_m)$ *versus* pH. Buffers employed were the same as described in Fig. 1. The *solid line* represents theoretically calculated values of log $(V_{max})/(K_m)$ at 25.5° (see text). The experimental points show the range of variation of five independent experiments at the same temperature. The *points of intersection of dotted lines* indicate $pK_{a,E}$ and $pK_{b,E}$ values at this temperature. The $pK_{a,E}$ and $pK_{b,E}$ values determined at other temperatures (shown in parentheses) are indicated by *arrows*. $V'_{max} = V_{max} \times 800$.

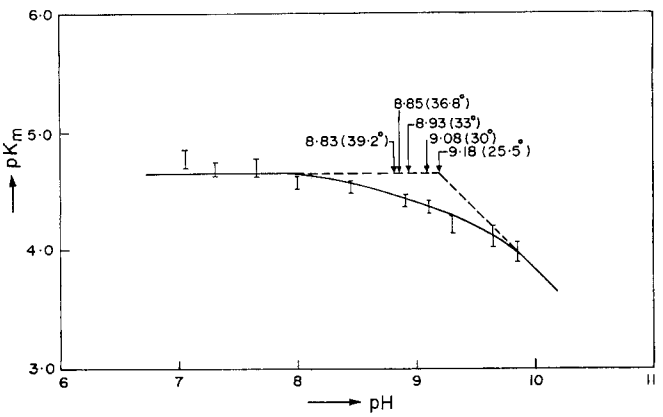

FIG. 3. Dixon plot of pK_m *versus* pH. Buffers employed were the same as described in Fig. 1. The *solid line* represents theoretically calculated values of pK_m at 25.5° (see text). The experimental points show the range of variation of five independent experiments at the same temperature. The *point of intersection of dotted lines* indicates $pK_{a,E}$ value at this temperature. The $pK_{a,E}$ values determined at other temperatures (shown in parentheses) are indicated by *arrows*.

using the following equation

$$pK_m = p\overline{K}_m - \log\left(1 + \frac{(H^+)}{K_{b,E}} + \frac{K_{a,E}}{(H^+)}\right) \quad (6)$$

where $K_{b,E}$ and $K_{a,E}$ represent the ionization constants of the free enzyme. This equation is derived from the equations of Dixon and Webb (18, 20) and assumes its present simplified form because of the absence in the graph of any pK values due to the free substrate or the enzyme-substrate complex. Since the experimental points do not indicate any ionization corresponding to the pK_b of the free enzyme, *i.e.* its $pK_{b,E}$, Equation 6 further simplifies to

$$pK_m = p\overline{K}_m - \log\left(1 + \frac{K_{a,E}}{(H^+)}\right) \quad (7)$$

from which the theoretical curve can be easily calculated. The theoretical curve is drawn as a *solid line* in Fig. 3. Visual inspection shows that the theoretical curve passes reasonably well through the experimental points, indicating the correctness of the $pK_{a,E}$ value within the limits of experimental error.

Since the pK_m *versus* pH curve shows only one inflexion corresponding to the $pK_{a,E}$ of 9.18 at 25.5°, it follows that there is no corresponding ionization of this group in the enzyme-substrate complex. Thus, it is obvious that it is this group in its acidic form which is involved in the link between the substrate and the enzyme and which thereby suffers a suppression in its ionization. The absence of any inflexion in the $pK_{b,E}$ region of this graph also shows that the state of ionization of the group represented by the $pK_{b,E}$ of 7.30 (Fig. 2) is the same in the free enzyme and in the enzyme-substrate complex (18, 20). In other words, as already shown above, the latter group participates only in catalytic mechanism and is not involved in binding between the enzyme and the substrate.

Fig. 3 also shows the effect of alteration of temperature on the $pK_{a,E}$ value of the enzyme. Using this data, the heat of ionization of the group corresponding to this pK has been calculated to be 11,000 cal. This value is the same as that obtained for this pK from a plot of log $(V_{max})/(K_m)$ against pH (Table I).

Effect of Organic Solvents on Kinetic Parameters—Findlay, Mathias, and Rabin (2) have developed a general method, based on the use of organic solvents, for identifying the charge types at the active site of an enzyme. This method follows from the fact that neutral acids increase their pK as water is replaced by an organic solvent such as dioxane or formamide, while cationic acids either decrease or have a constant pK under similar conditions. If one plots the activity of an enzyme in presence and in absence of the solvent against the pH, the neutral acid or cationic acid buffer solution would have in water alone (not in solvent-water mixture), it will be observed that the pH-activity curve in the organic solvent is displaced along the pH axis in a direction opposite to the change in pK, provided the effects on the ionizing groups of the protein are neglected. However, the effects due to the changes in the pK values of neutral or cationic acid groups at the active center of the enzyme run in opposite directions to those caused by the changes in the pK values of the buffers. The displacements in the pH-activity curves of the buffer and active center groups are additive and are summarized by Findlay *et al.* (see Table 1 in Reference 2). From the predicted direction of these displacements, it is possible to draw conclusions regarding the charge types at the active center of an enzyme.

Figs. 4 and 5 describe the effects of organic solvents on the kinetic parameters of alkaline phosphatase. It is to be noted that the pH values plotted in these figures are those obtained in

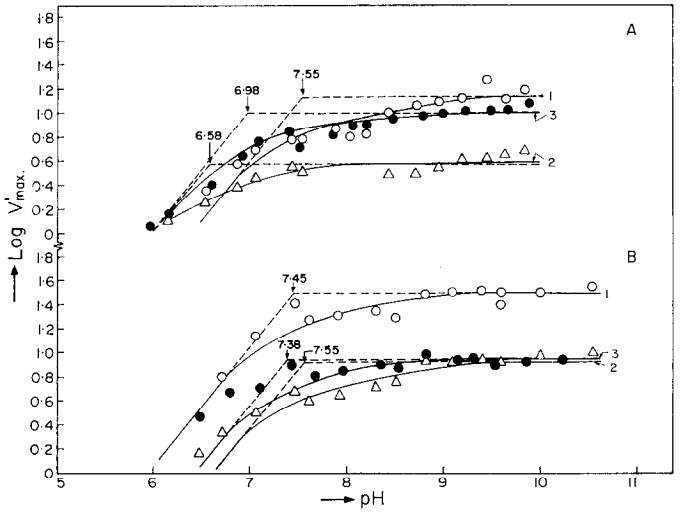

FIG. 4. Log V_{max} *versus* pH curves in a series of neutral acid buffers (*A*) and in a series of cationic acid buffers (*B*) in water (○, *curve 1*), in 25% (v/v) dioxane (△, *Curve 2*), and in 25% (v/v) formamide (●, *Curve 3*). The buffers employed were: *A*, pH 6.0 to 7.38, maleate; pH 7.52 to 9.90, phenol-*p*-sulfonate; *B*, pH 6.40 to 7.42, ethylenediamine hydrochloride; pH 7.60 to 8.52, *N*-ethylmorpholine hydrochloride; pH 8.80 to 10.95, ethylenediamine hydrochloride. The points were experimentally determined at 25° and the *solid curves* represent theoretically calculated values of log V_{max} at this temperature (see text). The *point of intersection of dotted lines* indicates $pK_{b,ES}$ value at this temperature. The pH values are those obtained in water and not in solvent-water mixtures. $V'_{max} = V_{max} \times 8000$.

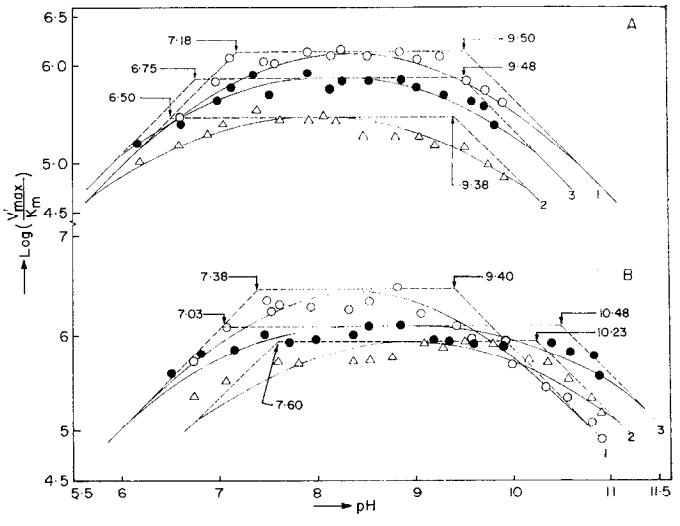

FIG. 5. Log $(V_{max})/(K_m)$ *versus* pH curves in a series of neutral acid buffers (*A*) and in a series of cationic acid buffers (*B*) in water (○, *Curve 1*), in 25% (v/v) dioxane (△, *Curve 2*), and in 25% (v/v) formamide (●, *Curve 3*). Buffers employed were the same as described in Fig. 4. The points were experimentally determined at 25° and the *solid curves* represent theoretically calculated values of log $(V_{max})/(K_m)$ at this temperature (see text). The *points of intersection of dotted lines* indicate $pK_{a,E}$ and $pK_{b,E}$ values at this temperature. The pH values are those obtained in water and not in solvent-water mixtures. $V'_{max} = V_{max} \times 8000$.

TABLE II

pK values determined in water, dioxane-water, and formamide-water

Solvent	$pK_{b,ES}$		$pK_{b,E}$		$pK_{a,E}$	
	Neutral acid buffers	Cationic acid buffers	Neutral acid buffers	Cationic acid buffers	Neutral acid buffers	Cationic acid buffers
Water	7.55	7.45	7.18	7.38	9.50	9.40
Dioxane-water	6.58	7.55	6.50	7.60	9.38	10.23
Formamide-water	6.98	7.38	6.75	7.03	9.48	10.48

water and not in solvent-water mixtures. Fig. 4*A* shows the log V_{max} *versus* pH plots for the enzyme in neutral acid buffers in water (*Curve 1*), 25% dioxane (*Curve 2*), and 25% formamide (*Curve 3*). These plots are then used to determine the effect of these solvents on the pK values of the ionizing groups on the enzyme-substrate complex responsible for its catalytic activity. It can be seen that in neutral acid buffers in both 25% dioxane and 25% formamide the $pK_{b,ES}$ of the enzyme shifted from 7.55 to 6.58 and 6.98, respectively. Similar results were obtained when the same experiments were repeated with 35% dioxane (not shown). On the other hand, Fig. 4*B* shows that the pK values in cationic acid buffers shifted from 7.45 in water (*Curve 1*) to 7.55 in 25% dioxane (*Curve 2*) and to 7.38 in 25% formamide (*Curve 3*). The considerable shift of $pK_{b,ES}$ obtained in neutral acid buffers and lack of a significant shift of this pK in cationic acid buffers show on the basis of predictions made by Findlay *et al.* (2) that a cationic acid group of pK around 7.50 is involved in catalysis.

The deviation between experimental points and theoretical lines (*Curve 1* in Fig. 4*A* and *Curves 1*, *2*, and *3* in Fig. 4*B*) can be partially attributed to the change in the rate-limiting step that occurs below pH 7.0. This has been explained above with reference to Fig. 1. *Curves 2* and *3* in Fig. 4*A* do not show this deviation because the actual pH values of neutral acid buffers in solvent-water mixtures are about 0.7 to 0.9 of a pH unit higher than those shown here and thus fall within the pH range in which there is only one rate-limiting step.

Fig. 5*A* shows the log $(V_{max})/(K_m)$ *versus* pH plot of this enzyme in neutral acid buffers in water (*Curve 1*), in 25% dioxane (*Curve 2*), and 25% formamide (*Curve 3*). These plots were then used to determine the effects of these solvents on the pK values of the ionizing groups on the free enzyme. It is clear that there is very little shift in the $pK_{a,E}$ of the enzyme in either 25% dioxane or 25% formamide. Fig. 5*B* describes a similar experiment in cationic acid buffers. Here the $pK_{a,E}$ has shifted from 9.40 in water (*Curve 1*) to 10.23 in 25% dioxane (*Curve 2*) and to 10.48 in 25% formamide (*Curve 3*). This lack of shift in neutral acid buffers and the considerable shift obtained in cationic acid buffers points to a neutral acid of about pK 9.4 as the one required for binding of the substrate to the enzyme.

Fig. 5*A* also shows that there is a significant shift in the $pK_{b,E}$ of the free enzyme in neutral acid buffers both in 25% dioxane and in 25% formamide. Here the $pK_{b,E}$ has shifted from 7.18 in water (*Curve 1*) to 6.50 in 25% dioxane (*Curve 2*) and to 6.75 in 25% formamide (*Curve 3*). On the other hand, Fig. 5*B* shows that the shifts produced in this pK in cationic acid buffers in either 25% dioxane or 25% formamide are relatively small. The significant shift of $pK_{b,E}$ in neutral acid buffers as against the small shift of this pK in cationic acid buffers enables one to conclude that a cationic acid group with a pK around 7.40 is involved in the catalytic mechanism. This conclusion thus agrees with the one drawn from Fig. 4.

These results are summarized in Table II.

DISCUSSION

Efforts to identify the amino acid or other residues involved in the catalytic function of an enzyme on the basis of pK values of ionizing groups derived from kinetic measurements and their heats of ionization are valid only if no conformational changes occur in the enzyme under the conditions of the experiment. If such changes occur, then it can be argued that an ionization which affects either V_{max} or K_m or both does so by changing the conformation of the enzyme and does not involve any residue which directly participates in the catalytic process. No such conformational changes have been shown to occur in the case of alkaline phosphatase molecule in the pH range 7.4 to 9.8 and in the temperature range 25–40°. Thus, absorption spectra of the enzyme in the 225 to 330 mμ range under these conditions (not shown) have been found to be identical and superimposable indicating lack of alteration either in the environment of aromatic residues or in the peptide backbone structure of the enzyme. Besides, in the present case only one ionization is observed in profiles of V_{max} and K_m with respect to pH. If as pointed out by Dyson and Noltmann (21) such ionizations are ascribed to conformational changes, then it is obligatory to assume for the group involved in enzyme-substrate binding that (*a*) either this group does not ionize, or (*b*) its ionization has no effect on binding, or (*c*) it ionizes outside the pH range in which kinetic studies are carried out. Since these assumptions are not reasonable, one expects to observe distinct pK values relating to both conformational changes and to groups involved in enzyme-substrate binding. In the present case, only one inflexion each was observed in profiles of log V_{max} and K_m with respect to pH. It is therefore much easier to identify these inflexions with the ionizable groups involved in the catalytic process.

Likewise, caution is necessary in interpreting shifts in pK values of active center groups in presence of organic solvents. Thus, organic solvents can bring about conformational changes in the structure of a protein and can also affect the activated state of the enzyme-substrate complex beside decreasing water concentration which is of relevance in a hydrolytic reaction such as the one catalyzed by alkaline phosphatase (2). Some of these effects, especially changes in enzyme configuration, can produce shifts in pK values, the magnitude and direction of which can obscure those expected from studies on small molecules (22). In our experiments, several lines of evidence indicate that such conformational changes could not have occurred in alkaline phosphatase under the conditions of solvent composition employed. Thus, the minimum K_m value for the enzyme in the pH range studied was the same in 25% dioxane and 25% formamide as in water. It is difficult to visualize a significant conformational change in an enzyme which could have no effect on its affinity with the substrate. Besides, dioxane and formamide are quite dissimilar solvents in the way they cause denaturation in proteins. Whereas dioxane acts by dissolving hydrophobic structures in a protein, formamide probably denatures because of its high dielectric constant (23). Hence, conformational changes in the enzyme produced by these solvents will not necessarily produce similar shifts in the pK values of active center groups. However, in the case of alkaline phosphatase

both the ionization constants of the enzyme undergo shifts in directions which are the same for either of these solvents. This result constitutes significant evidence against the possibility of a conformational change in the enzyme vitiating interpretation of pK shifts in these two organic solvents. Lastly, the most direct evidence for the lack of any significant conformational changes in these solvents comes from difference spectral studies (not shown). No differences were observed between the absorption spectra of the enzyme in solvent-water mixtures and in water in the 250 to 350 mμ range indicating absence of any change in the environment of aromatic residues of the enzyme in the presence of organic solvents. For technical reasons, these spectral studies could not be extended to the far ultraviolet region. However, absence of disturbance in the environment of aromatic chromophores in solvent-water mixtures, along with other evidence detailed above, strongly indicates that the direction of shifts in pK values in organic solvents could be safely attributed to the charge types at the active center and not to denaturation in presence of organic solvents. The lower activity of the enzyme at all pH values in presence of organic solvents (Fig. 4) is probably due to the reduction in the activity of water which is bound to affect a hydrolytic reaction.

pK_b Value of Enzyme-Substrate Complex—On the basis of the $pK_{b,ES}$ value of 7.43 at 25.5°, it is possible to conclude as a first approximation, that the imidazole group of a histidine residue is involved in the catalytic hydrolysis of *p*-nitrophenyl phosphate by *E. coli* alkaline phosphatase. Although in a few rare instances side chain carboxyls have also been found to exhibit high pK values of 7.4, these are known to be abnormal groups unavailable to the solvent, which on denaturation of the protein titrate normally (24). Such groups cannot therefore serve as models in the present case. The effect of organic solvents on kinetic parameters also indicated that the group under consideration was cationic in nature. In addition, the fact that the heat of ionization of this group is 6,500 cal rules out the participation of a carboxyl group (heat of ionization ±1,500 cal) in the catalytic mechanism. The only other group beside imidazole that deserves serious consideration is a terminal α-amino group which, on the basis of data on small molecules, is expected to have a pK of 7.8 (24) and is also cationic. At a first glance, α-amino group can be ruled out on the basis of its heat of ionization of 10,000 to 13,000 cal (18) which is high compared to the value of 6,500 cal obtained in the present case. Lindley (25) has pointed out, however, that when two ionizing groups are in close proximity to each other, each of them tends to yield a value for the heat of ionization which is midway between the values normally found for either of them depending on how closely the pK values of the two groups approach each other. Thus, the presence of a charged carboxyl group close to an α-amino group would depress the heat of ionization of the α-amino group to about 6,000 cal and raise that of carboxyl group to about the same value. But in such a case, the pK value exhibited by the α-amino group would be much higher than 7.8, due to the electrostatic effect (26). It is therefore improbable that the $pK_{b,ES}$ values of 7.43 obtained in the present case would correspond to an α-amino group. The choice thus narrows down to the imidazole group of histidine, as controlling the rate-limiting step in the sequence of reactions resulting in the catalytic hydrolysis of *p*-nitrophenyl phosphate by *E. coli* alkaline phosphatase.

Lazdunski and Lazdunski (4) who reached similar conclusion implicated this imidazole group also in the binding of the substrate. They arrived at this finding apparently on the basis of their observation that the pK of this group shifts from 7.1 in the free enzyme to 7.4 in the enzyme-substrate complex, with a change in its heat of ionization from 6600 cal in the free enzyme to 8700 cal in the enzyme-substrate complex. We have not observed any significant difference in the pK of this group in the free enzyme and in the enzyme-substrate complex (Figs. 1 and 2) nor in its heat of ionization in these two states (Table I). Besides, our pK_m *versus* pH curve (Fig. 3) shows no bends in this region convex to the pH axis, indicating thereby that the ionization of this group does not change on binding of the substrate to the enzyme. Such a bend is also absent in the pK_m *versus* pH curve described by Lazdunski and Lazdunski (4). It is thus safer to conclude that the shift of 0.3 of a pK unit which they observed for this group in the enzyme-substrate complex was probably an experimental variation and did not signify any role for the imidazole group in the binding of the substrate.

No other evidence beside kinetic has so far been presented for the involvement of histidine in the catalytic process. Plotch and Lukton (27) failed to show any significant effect of iodoacetic acid treatment or photooxidation, on the activity of the enzyme. Tait and Vallee (28), on the basis of photooxidation studies on the holoenzyme and apoenzyme of alkaline phosphatase, concluded that some histidines were liganded to zinc atoms of the enzyme. However, the histidine postulated here as participating in catalysis cannot be identified with any of the histidines liganded to zinc. For, in such a case the pK of the histidine would have been much lower than observed. Also if this histidine is liganded to one of the zinc atoms, then its protonation would probably lead to a conformational change in the enzyme which was not detected in the present case. Therefore, more work is needed before one can confidently implicate a histidine in the catalytic mechanism.

pK_a Value for Free Enzyme—Several amino acid residues can be considered for assignment to the $pK_{a,E}$ value of 9.18 at 25.5° which represents a group involved directly or indirectly in enzyme-substrate binding. These are the terminal α-amino group, ε-amino group of lysine, the phenolic hydroxyl group of tyrosine, and the sulfhydryl group of cysteine.

The sulfhydryl group of cysteine can be easily disregarded since the enzyme lacks free sulfhydryl groups (29). The pK of 9.18 is too high to correspond to an α-amino group unless this group exists in a salt bridge with a negatively charged carboxyl group. But in such a case its heat of ionization would tend to be lower than the observed value of 11,000 cal (25). In addition, the effect of organic solvents on kinetic parameters shows that the group having a $pK_{a,E}$ of 9.18 is a neutral acid. All of these factors would thus rule out α-amino group as the one involved in binding. The phenolic hydroxyl group of tyrosine, although a neutral acid, cannot be considered for this assignment, as the absorption spectra of the enzyme in the pH range 6.8 to 9.8 failed to show any ionization of tyrosine groups.

The expected value for the ionization of ε-amino group of lysine in proteins is 10.4. However, in several proteins, these groups ionize at pK values about 0.5 to 1.0 unit lower than the expected (24). Thus, a group with the observed $pK_{a,E}$ of 9.18 together with a heat of ionization of 11,000 cal could very well be the ε-amino group of lysine. But as pointed out above, the group under consideration is a neutral acid and not a cationic

acid. This would make it unlikely that the ϵ-amino group of lysine is the group involved in enzyme-substrate binding.

Since no ionizable side chain of an amino acid residue is apparently involved in binding of the substrate, it is worth considering the role of the metal in this respect. Alkaline phosphatase of *E. coli* is a zinc metalloprotein (15). Zinc is essential for the activity of the enzyme but can be replaced by cobalt (30). A water molecule in the coordination sphere of a zinc atom would dissociate a proton as follows:

$$Zn^{++}(H_2O) \rightleftarrows Zn^{++}(OH)^- + H^+$$

A water molecule coordinated to the zinc atom has a pK of 8.96 at 25° and a heat of ionization of about 13,000 cal (31). Besides, it is a neutral acid. Thus, it has all of the properties which we know are associated with the group involved in enzyme-substrate binding. We therefore propose that a water molecule coordinated to the zinc atom is the positively charged center which binds the negatively charged phosphate of the substrate. Simpson and Vallee (32), on the basis of spectral studies carried out on alkaline phosphatase in which zinc was replaced by cobalt, concluded that inorganic phosphate, a competitive inhibitor of the enzyme, binds to the enzyme in close vicinity to the metal. Gottesman *et al.* (6) have recently carried out kinetic studies on cobalt alkaline phosphatase. On the basis of results similar to the ones we have described here, they speculate on the possibility of a zinc-water coordination complex being the binding site of the enzyme. An analysis of their data which extends to the high pH region shows that although the affinity of their enzyme for the substrate decreases with increasing pH, there is still some residual affinity at very high pH values. This fact agrees very well with the proposal that zinc-water coordination complex is the substrate-binding site on the enzyme. For even after the dissociation of a proton, this complex still has a unit positive charge to bind the negatively charged phosphate of the substrate.

REFERENCES

1. Herries, D. G., Mathias, A. P., and Rabin, B. R., *Biochem. J.*, **85,** 127 (1962).
2. Findlay, D., Mathias, A. P., and Rabin, B. R., *Biochem. J.*, **85,** 139 (1962).
3. Findlay, D., Herries, D. G., Mathias, A. P., Rabin, B. R., and Ross, C. A., *Biochem. J.*, **85,** 152 (1962).
4. Lazdunski, C., and Lazdunski, M., *Biochem. Biophys. Acta*, **113,** 551 (1966).
5. Lazdunski, C., and Lazdunski, M., *Biochim. Biophys. Acta*, **147,** 280 (1967).
6. Gottesman, M., Simpson, R. T., and Vallee, B. L., *Biochemistry*, **8,** 3776 (1969).
7. Schwartz, J. H., and Lipmann, F., *Proc. Nat. Acad. Sci., U. S. A.*, **47,** 1996 (1961).
8. Engström, L., *Biochim. Biophys. Acta*, **56,** 606 (1962).
9. Barret, H., Butler, R., and Wilson, I. B., *Biochemistry*, **8,** 1042 (1969).
10. Schwartz, J. H., *Proc. Nat. Acad. Sci. U. S. A.*, **49,** 871 (1963).
11. Aldridge, W. N., Barman, T. E., and Gutfreund, H., *Biochem. J.*, **92,** 23C (1964).
12. Fernley, H. N., and Walker, P. G., *Nature*, **212,** 1435 (1966).
13. Halford, S. E., Bennett, N. G., Trentham, D. R., and Gutfreund, H., *Biochem. J.*, **114,** 243 (1969).
14. Malamy, M., and Horecker, B. L., in S. P. Colowick and N. O. Kaplan (Editors), *Methods in enzymology, Vol. IX*, Academic Press, New York, 1966, p. 639.
15. Plocke, D. J., Levinthal, C., and Vallee, B. L., *Biochemistry*, **1,** 373 (1962).
16. Lineweaver, H., and Burk, D., *J. Amer. Chem. Soc.*, **56,** 658 (1934).
17. Bacarella, A. L., Grunwald, E., Marshall, H. P., and Purlee, E. L., *J. Phys. Chem.*, **62,** 856 (1958).
18. Dixon, M., and Webb, E. C., *Enzymes*, Ed. 2, Longmans, Green and Company, Ltd., London, 1964, p. 144.
19. Delory, G. E., and King, E. J., *Biochem. J.*, **37,** 547 (1943).
20. Dixon, M., *Biochem. J.*, **55,** 161 (1953).
21. Dyson, J. E. D., and Noltmann, E. A., *J. Biol. Chem.*, **243,** 1401 (1968).
22. Gutfreund, H., *An introduction to the study of enzymes*, Blackwell Scientific Publications, Oxford, 1965, p. 106.
23. Singer, S. J., *Advan. Protein Chem.*, **17,** 1 (1962).
24. Steinhardt, J., and Beychok, S., in H. Neurath (Editor), *The proteins, Vol. II*, Ed. 2, Academic Press, New York, 1964, p. 139.
25. Lindley, H., *Biochim. Biophys. Acta*, **59,** 235 (1962).
26. Tanford, C., *Advan. Protein Chem.*, **17,** 69 (1962).
27. Plotch, S., and Lukton, A., *Biochim. Biophys. Acta*, **99,** 181 (1965).
28. Tait, G. H., and Vallee, B. L., *Proc. Nat. Acad. Sci. U. S. A.*, **56,** 1247 (1966).
29. Schlesinger, M. J., and Barrett, K., *J. Biol. Chem.*, **240,** 4284 (1965).
30. Plocke, D. J., and Vallee, B. L., *Biochemistry*, **1,** 1039 (1962).
31. Perrin, D. D., *J. Chem. Soc.*, 4500 (1962).
32. Simpson, R. T., and Vallee, B. L., *Biochemistry*, **7,** 4343 (1968).