Chicken Intestinal Alkaline Phosphatase

I. *The kinetics and thermodynamics of reversible inactivation*

II. *Reactivation by zinc ions*

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ABSTRACT Purified chicken intestinal alkaline phosphatase is active at pH 8 to 9, but becomes rapidly inactivated with change of pH to 6 or less. Also, a solution of the inactivated enzyme at pH 4.5 rapidly regains its activity at pH 8. In the range of pH 6 to 8 a solution of purified alkaline phosphatase consists of a mixture of active and inactive enzyme in equilibrium with each other. The rate of inactivation at lower pH and of reactivation at higher pH increases with increase in temperature. Also, the activity at equilibrium in the range of pH 6 to 8 increases with temperature so that a solution equilibrated at higher temperature loses part of its activity on cooling, and *vice versa*, a rise in temperature shifts the equilibrium toward higher activity. The kinetics of inactivation of the enzyme at lower pH and the reactivation at higher pH is that of a unimolecular reaction. The thermodynamic values for the heat and entropy of the reversible inactivation and reactivation of the enzyme are considerably lower than those observed for the reversible denaturation of proteins. The inactivated enzyme at pH 4 to 6 is rapidly reactivated on addition of Zn ions even at pH 4 to 6. However, zinc ions are unable to replace magnesium ions as cocatalysts for the enzymatic hydrolysis of organic phosphates by alkaline phosphatase.

INTRODUCTION

Alkaline phosphatase, as its name implies, catalyzes the hydrolysis of organic phosphate esters in alkaline medium, at an optimum pH of about 9.0, in the presence of magnesium or manganese ions.

Alkaline phosphatase is found in various organs and organisms, the most common sources being yeast, animal bone, kidney, and intestinal epithelium.

Crude extracts of the enzyme can be purified by fractional precipitation with ammonium sulfate and alcohol, and also by adsorption on aluminum hydroxide or calcium phosphate gels. The purified materials are less stable

Received for publication, December 8, 1959.

than the crude extracts, the stability depending greatly on the pH of the medium. Thus, Martland and Robison, as early as 1927, observed that purified alkaline bone phosphatase loses its enzymatic activity when stored at pH 6.0 or lower. This was also confirmed by Bauer in 1936 who found that purified alkaline yeast phosphatase at pH 6.0 lost most of its activity when incubated for 10 minutes at 40°C.

In 1938 Albers and coworkers, while working with alkaline kidney phosphatase, discovered that the inactivation brought about in purified preparations of the enzyme at pH 5.0 is partly regained when the pH of the solution is raised to 8.4 (Shales and Mann (1948)).

Cloetens from 1942 to 1944 carried out an extensive study of the effect of various salts and other reagents on the rate of inactivation in acid medium of purified kidney phosphatase and of its reactivation in alkaline medium. Cloetens demonstrated that inactivation in acid medium is nearly completely prevented in the presence of zinc ions; the inactivation was also partly prevented by vanadyl (VO) ions.

The present writer, while working on the purification of chicken intestinal phosphatase, observed that crude preparations of the enzyme on fractionation with ammonium sulfate gradually lose a great deal of activity. The lost activity is, however, regained on adjusting the pH of the inactivated material to 8.0 or higher. The reactivated solutions gradually lose their activities again on acidification to pH 5.0. This phenomenon of reversible inactivation and reactivation of an enzyme by simply shifting the pH one or two units from the point of neutrality is highly unusual.[1] A study was therefore undertaken of the kinetics and thermodynamics of the reversible reaction with the object of finding a possible clue to the mechanism of this phenomenon.

EXPERIMENTAL TECHNIQUE

Preparation of Purified Alkaline Phosphatase

The starting material was a crude commercial preparation of alkaline phosphatase (Worthington) obtained from a butanol-water extract of chicken intestines and partly purified by fractional precipitation between 0.4 and 0.6 saturation of ammonium sulfate. The commercial material was refractionated in the writer's laboratory between 0.4 and 0.7 saturation of ammonium sulfate and further purified by fractionation with ethanol and adsorption on calcium phosphate gel, followed by elution with dilute ammonium sulfate solution, pH 8.5, and refractionation with ethanol. The procedure is as follows:—

1. FRACTIONATION WITH ETHANOL. ALBERS (1935), PORTMANN (1957) 20 gm. filter cake (0.7 s.a.s. fraction) is dissolved in 200 ml. water at 3–5°C. The solution is

[1] Northrop, in 1955, observed reversible inactivation and reactivation of a bacteriophage by simply changing the pH of the phage solution back and forth between pH 6 and 7.

titrated with 0.5 N H_2SO_4 to pH 5.0. 200 ml. 95 per cent ethyl alcohol is then added slowly with stirring while the temperature of the solution is allowed to drop gradually to about −5°C. The precipitate formed—1st alcohol precipitate—is removed by centrifugation at −8°C. and is rejected. A second portion of 200 ml. of 95 per cent alcohol is then added slowly with stirring to the supernatant solution at −8°C. The mixture is stored for 1 hour at −8°C. and then centrifuged at the same temperature. The residue—2nd alcohol precipitate—containing most of the original phosphatase activity is suspended in about 60 ml. cold water, centrifuged clear at 5°C., and is washed by centrifugation with 30 ml. cold water.

2. ADSORPTION ON CALCIUM PHOSPHATE GEL The combined supernatant solutions are mixed with 50 ml. of stock of cold calcium phosphate gel (Kunitz (1952)) and centrifuged at 5°C. The residue is stirred up with 10 gm. highflo-supercel in about 50 ml. water and recentrifuged. The final residue is suspended in about 20 ml. 0.033 saturated $(NH_4)_2SO_4$ pH 8.5 (NH_4OH), filtered with suction on soft filter paper, and washed repeatedly with 10 to 15 ml. of eluant until the optical density at 280 mμ of the filtrate is less than 0.5. The combined filtrates are titrated with 0.5 N H_2SO_4 to pH 5.0.

3. REFRACTIONATION WITH ALCOHOL. DRYING WITH ACETONE The calcium phosphate eluate is refractionated with alcohol as described in step 1, except that the "2nd alcohol" residue is resuspended not in water but in a small amount of 65 per cent alcohol at −8°C., transferred to a small centrifuge tube, and recentrifuged at −8°C. The final residue is stirred up again with 2 ml. of 65 per cent alcohol and poured into 75 ml. acetone at −8°C. with stirring. The mixture is filtered immediately with suction on 5 cm. hardened paper, washed with cold acetone, and dried on the filter paper. The dry material is ground fine in a mortar, then allowed to dry in the room for several hours, and stored at 5°C.

The yield is about 200 mg. of dry powder per 20 gm. of 0.7 s.a.s. filter cake of a specific activity (after activation) of about 15,000 units per mg. dry material, as compared with about 500 units per mg. of the commercial starting material. The nearly colorless dry powder is readily soluble in 0.1 M tris buffer, pH 8.5. At pH 4.5 (0.1 M acetate buffer) the powder dissolves more slowly yielding a slightly turbid solution which can be clarified by centrifugation.

The dried preparation possesses no phosphatase activity when brought into solution either at pH 4.5 or at pH 8.5 but becomes rapidly active at pH 8.5, while in solution at pH 4.5 it remains inactive unless reactivated by change of pH.

Stock solutions of the purified enzyme in 0.1 M acetate buffer, pH 4.5, or in 0.1 M tris buffer, pH 8.5, are stable for a week or longer when stored at 5°C. The purified preparations have been used for most of the studies reported here. Several studies were also made with preparations of lesser purity. The main characteristic of the phenomenon of reversible inactivation and reactivation is also apparent in crude preparations of the enzyme but becomes more pronounced in preparations of higher purity.

PHOSPHATASE ACTIVITY MEASUREMENT. BRANDENBERG and HANSON (1953), HOFSTEE (1954) Phosphatase activity was measured by determining the catalytic

effect of the enzyme on the initial rate of hydrolysis of a dilute solution of the phosphate ester of salicylic acid (*o*-carboxyphenyl phosphate) in glycine buffer, pH 8.8. The liberated salicylic acid, unlike the phosphate ester, absorbs ultraviolet light at about 300 mμ; the rate of hydrolysis can thus be followed spectrophotometrically, one unit of phosphatase being equal to an increase in optical density at 300 mμ of 0.001 per minute at 25°C.

ASSAYING MIXTURE: PROCEDURE

2 ml. 0.2 M glycine buffer pH 8.8
1 ml. 0.00365 M *o*-carboxyphenyl phosphate (20 mg./25 ml. H_2O)
0.5 ml. 0.05 M $MgCl_2$

The components are mixed in 15 ml. pyrex test tubes, warmed to 25°C., then 0.1 ml. of enzyme sample is added, mixed, and transferred to a quartz cuvette. Optical density at 300 mμ is measured at 25°C. at half-minute intervals for 3 minutes against a blank made up in the same way but without enzyme. The plotted values of optical density *vs.* time in case of fully activated samples of enzyme generally form straight lines, the slopes of which are proportional within a wide range to the concentrations of the enzyme used.

Measurement of Rate of Inactivation and of Reactivation

(A) PROCEDURE Samples of stock solutions of purified enzyme in 0.1 M acetate buffer, pH 4.5, or in 0.1 M tris buffer, pH 8.5, and containing about 1 mg. dry material per ml., were diluted tenfold in glass-stoppered pyrex test tubes with 0.05 or 0.1 M acetate or tris buffer solutions of various pH, previously heated to the required temperature, and left in a constant temperature water bath. Samples of 0.1 ml. of the diluted solutions were then added at various times to 3.5 ml. of the standard assaying mixture for activity measurements.

(B) REACTIVATION IN THE ASSAYING MIXTURE DURING THE MEASUREMENT OF ACTIVITY The study of the rate of inactivation or reactivation of an enzyme involves the problem of finding the proper conditions for assaying the enzyme so as to avoid a change in its activity during the assay. In the case of alkaline phosphatase a certain amount of reactivation does occur during the assaying of the partly inactivated enzyme. The rate of reactivation, however, depends greatly on the chemical nature of the buffer component of the assaying mixture. Reactivation is very slight in the presence of glycine buffer. This is shown in Fig. 1 where the curves for the rate of hydrolysis of the substrate by partly inactivated enzyme in the presence of either glycine or tris buffer are given; the difference between the rapidly rising S-shaped curve in the case of the tris buffer, and the nearly straight line yielded in the presence of glycine of the same pH and concentration is striking. Entirely different curves were obtained when the hydrolysis of the phosphate ester was carried out by samples of fully reactivated enzyme. This is shown in Fig. 2. The hydrolysis was more rapid in the presence of tris buffer than in the presence

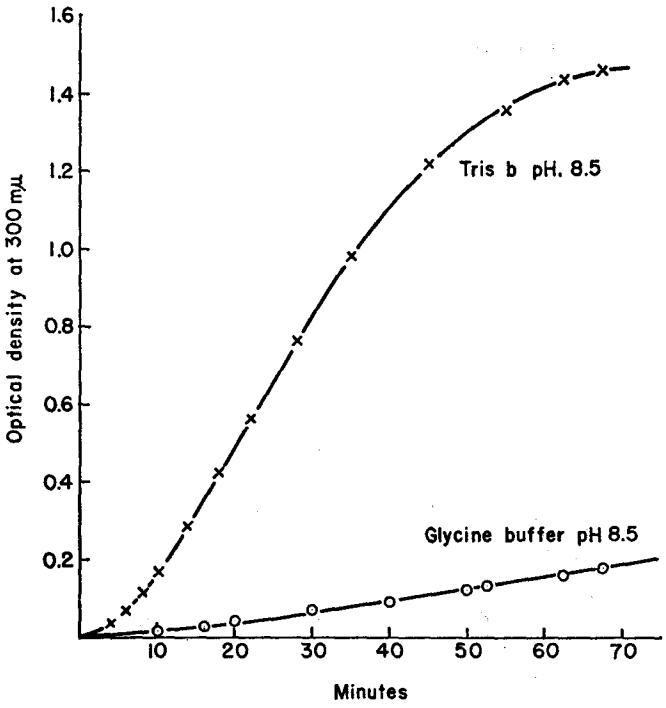

FIGURE 1. Reactivation of purified chicken alkaline phosphatase in tris buffer, pH 8.5, during the assaying of the enzyme.

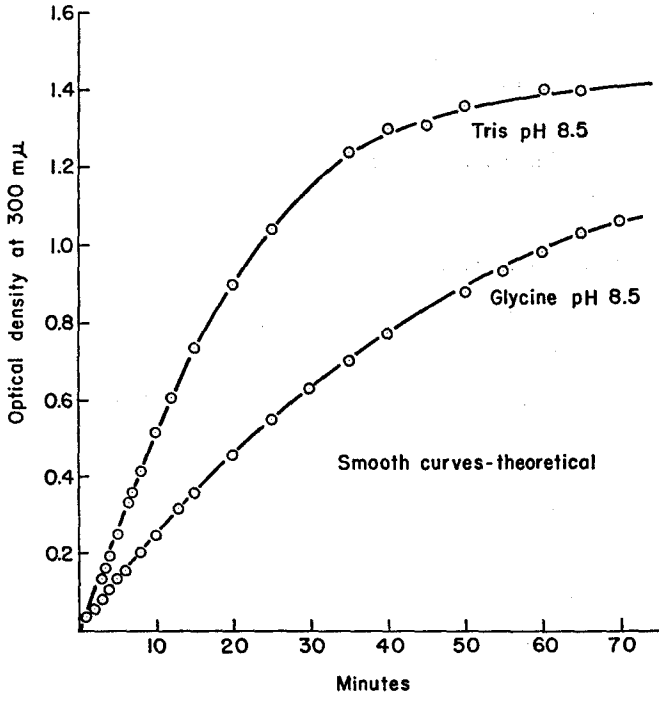

FIGURE 2. Effect of tris and glycine buffer solutions, pH 8.5, on rate of hydrolysis of *o*-carboxyphenyl phosphate by alkaline phosphatase.

of glycine; the curves, however, were of the same unimolecular type differing only in the values of the velocity constants, being 0.02 per minute in the glycine buffer and 0.05 per minute in tris buffer.

But even in the presence of glycine there was usually a significant amount of reactivation during the first 3 minutes of assaying, giving rise to slightly concave curves, instead of the straight lines generally obtained with fully activated samples of enzyme: the activity was then determined by measuring the slopes of the lines drawn tangentially to the curves at zero time.

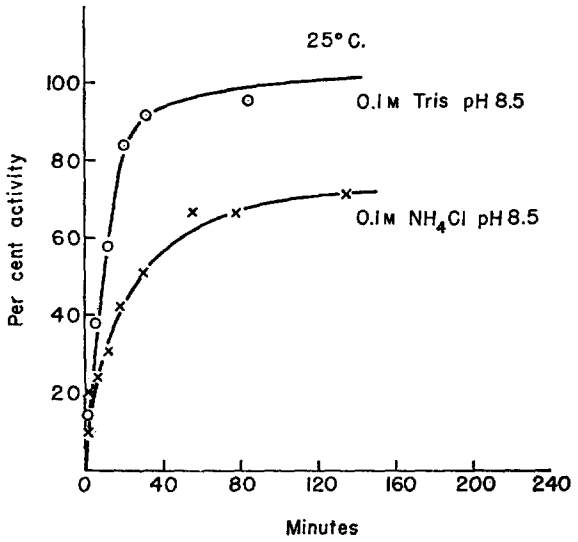

FIGURE 3. Effect of type of buffer solution, pH 8.5, on rate of reactivation of alkaline phosphatase at 25°C.

EXPERIMENTAL RESULTS

The results of a series of studies on the stability of purified chicken intestinal alkaline phosphatase at various pH and temperatures are as follows:

1. *Effect of pH* Rapid and nearly complete inactivation occurs when a solution of active phosphatase of pH 8.5 is brought to the range of pH 4 to 6. On the other hand, a solution of inactivated enzyme rapidly regains its activity when brought to the range of pH 8 or 9. It loses its activity again when brought back to the acid range of pH.

In the intermediate range of pH of about 6 to 8 a solution of purified alkaline phosphatase consists of a mixture of active and inactive enzyme in equilibrium with each other in proportions varying with the pH, temperature, and type of buffer used, the final equilibrium being approximately the same whether the material was fully active or inactive when brought to the range of pH 6 to 8.

2. *Effect of Temperature.* In the range of temperatures of 5 to 35°C. the rate of inactivation at a lower pH and of reactivation at a higher pH increases with increase in temperature. Also, the equilibrium values a_e in the range of pH 6 to 8 have a tendency to increase with rise in temperature. Thus, a solution of partly activated material at a higher temperature will

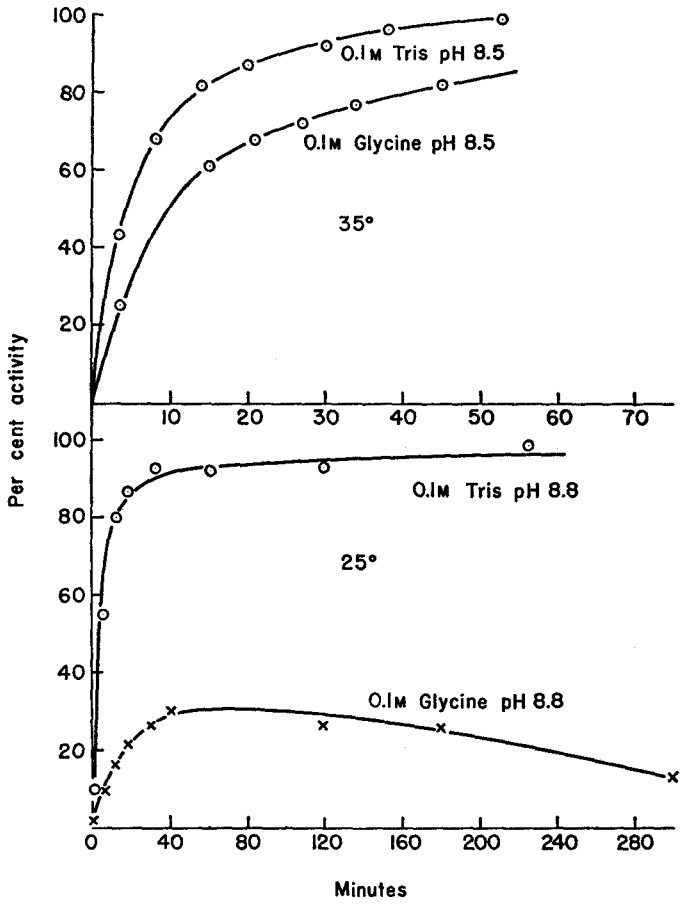

FIGURE 4. Effect of tris and glycine buffer solutions, pH 8.5 and 8.8, on the reactivation of alkaline phosphatase.

gradually lose part of its activity when stored at a lower temperature, and it will regain its lost activity when brought back to the higher temperature. This is the reverse of the behavior of most enzymes on reversible inactivation by heat, the enzymes generally losing activity at higher temperatures and regaining activity as the temperature is lowered.

3. *Effect of Buffer Solution on Reactivation* The rate as well as the final value of reactivation at pH 8 to 9 depends greatly on the chemical composition

of the buffer solution used. Tris buffer[2] in concentrations of 0.1 to 0.05 was found to be most effective as a reactivator with respect to both the rate and the equilibrium value of the reactivation (Figs. 3 and 4).

I. THE KINETICS AND THERMODYNAMICS OF REVERSIBLE INACTIVATION

Kinetics Both processes appear to follow closely the course of unimolecular reactions as shown by the plotted curves of activity *vs.* time. See Figs. 5 and 6. This is confirmed by the fact that within the range of concentrations of enzyme used the rate of *percentage* change in both reactions appears to be independent of concentration. See Figs. 7 and 8.

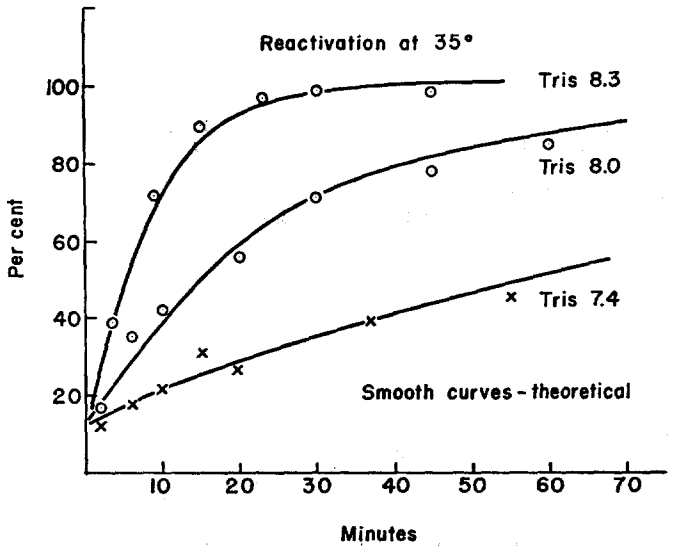

FIGURE 5. Rate of reactivation of alkaline phosphatase at 35°C. in the presence of 0.1 M tris buffer of various pH.

The smooth theoretical curves in Figs. 5 to 8 were plotted in accordance with the following equations (see Kunitz, 1948):

$$a = 100\, e^{-k_1 t} \text{ for inactivation, in the range of pH 4 to 6} \qquad (1)$$

and

[2] The pH of tris buffer solutions decreases somewhat with temperature and had to be determined at the temperatures used, against a standard of known temperature coefficient, Beckman standard pH 7.0, diluted 25-fold.

$a = 100 - 100\ e^{-k_2 t}$ for reactivation, in the range of pH 8 to 9, a being expressed in per cent of total enzyme concentration. (2)

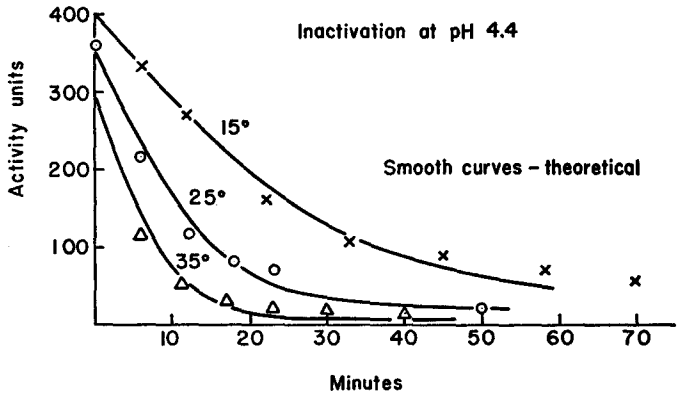

FIGURE 6. Rate of inactivation of alkaline phosphatase at pH 4.4 and various temperatures.

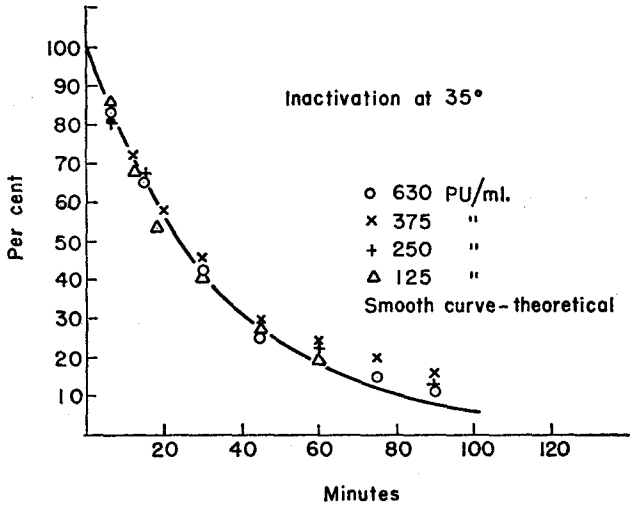

FIGURE 7. Rate of inactivation of various concentrations of alkaline phosphatase at pH 4.5 and 35°C.

The velocity constants k_1 and k_2 per second were obtained from the relationship

$$k = \frac{2.3 \log 2}{t_2} = \frac{0.69}{t_2}$$

where t_2 is the time in seconds required for 50 per cent inactivation or reactivation. This approximate method of obtaining the velocity constants was found adequate for the general precision of these studies.

In the range of pH 6 to 7 both reactions proceed at a slow rate, making it difficult to determine the exact values of a_e, k_1, k_2, and K_e. However, it was found possible to estimate these values by extrapolation, as described in the following section.

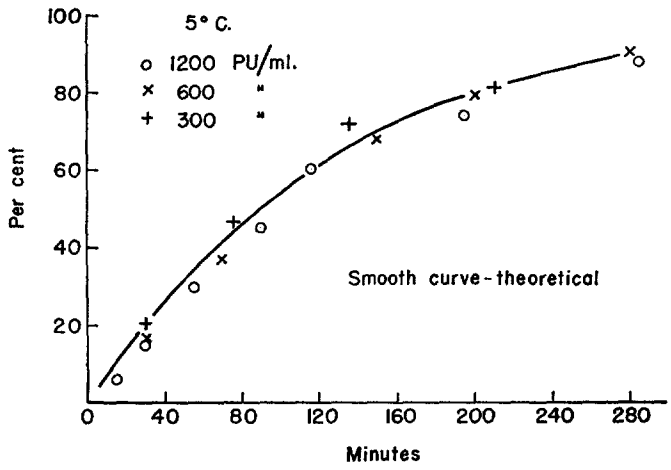

FIGURE 8. Rate of reactivation of various concentrations of alkaline phosphatase at pH 8.5 and 5°C.

The Empirical Relationship between the Velocity Constants, pH, and Temperature

(A) INACTIVATION Fig. 9 shows a series of isothermal parallel straight lines drawn through the plotted experimental values of pk_1—the negative logs of k_1—*vs.* pH in the range of pH 4.4 to 5.6, and in the range of temperatures of 5 to 35°C. The slope of the parallel lines is equal to 0.5, while the intercepts pC_1 decrease with rise in temperature.

The general equation for the straight lines is:

$$pk_1 = 0.5\ p\mathrm{H} + pC_1 \tag{3}$$

Hence

$$k_1 = C_1[\mathrm{H}^+]^{0.5} \tag{4}$$

which means that the percentage rate of inactivation of the enzyme at constant temperature in the range of 5 to 35°C. is proportional to the square root of the hydrogen ion concentration, the proportionality constant C_1 being

independent of pH but increasing with temperature. The temperature coefficient Q_{10} for any interval T_2-T_1 of 10°C. equals the ratio of the values of C_1 for the corresponding temperatures. The values of pC_1, C_1, and Q_{10} are given

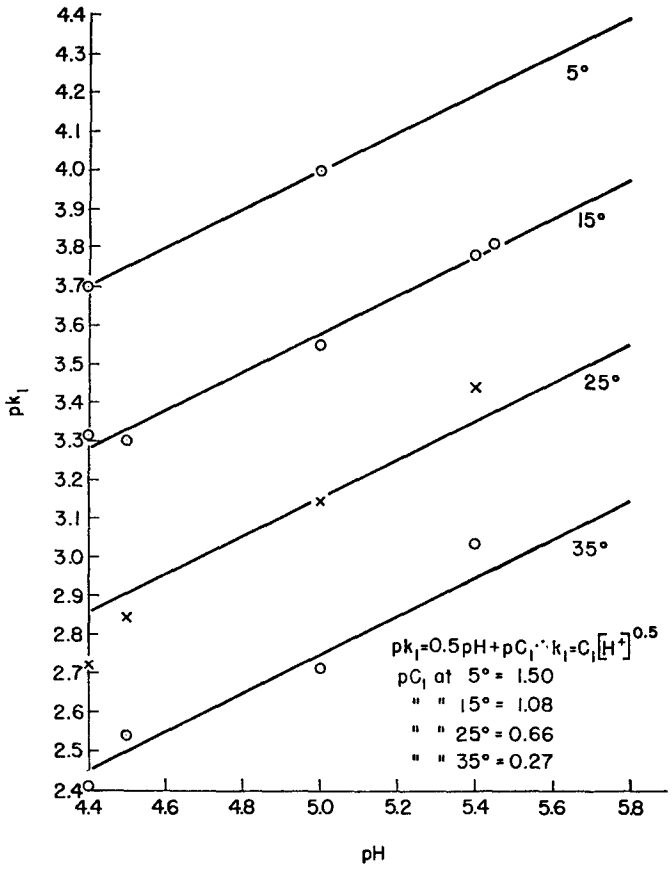

FIGURE 9. Plotted values of pk_1 *vs.* pH for various temperatures.

TABLE I

REACTION: ACTIVE → INACTIVE; $pk_1 = 0.5\ pH + pC_1$

Temperature.....	5°C.	15°C.	25°C.	35°C.
pC_1.....	1.50	1.08	0.66	0.27
C_1.....	0.0316	0.083	0.224	0.540
Q_{10}.....	2.62	2.70		2.41

in Table I. The average value of Q_{10} for inactivation is only 2.5 as compared with the value of about 10 generally observed for denaturation of proteins by heat.

(B) REACTIVATION Parallel isothermal straight lines were also obtained when the values of pk_2 were plotted against pH in the range of pH 7.4 to 8.9, for the temperatures of 5 to 35°C. (see Fig. 10). The slope of the lines is equal to -1.0 and the general equation is:

$$pk_2 = -p\mathrm{H} + pC_2 \tag{5}$$

Hence

$$k_2 = \frac{C_2}{[\mathrm{H}^+]} \tag{6}$$

Thus, the percentage rate of reactivation of the enzyme at constant temperature in the range of 5 to 35°C., is inversely proportional to the hydrogen ion concentration. The proportionality constant C_2 is independent of the pH of solution but increases with increase in temperature. The values of the constants C_2 for the various temperatures and also the values of Q_{10} are given in Table II. The values for Q_{10} for reactivation are significantly higher than those for inactivation.

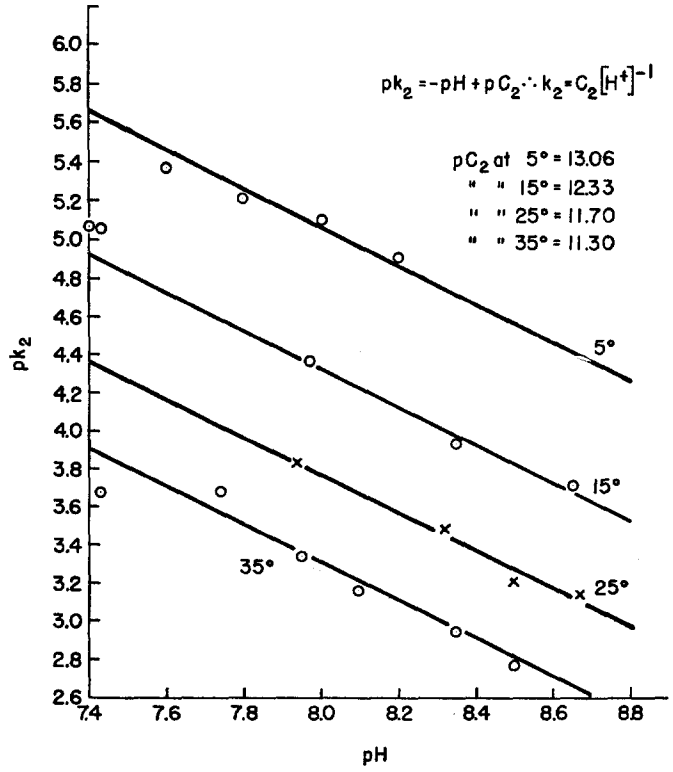

FIGURE 10. Plotted values of pk_2 *vs.* pH for various temperatures.

THE POSSIBLE EFFECT OF CONCENTRATION OF HYDROXYL IONS ON THE RATE OF REACTIVATION The relationship

$$k_2 = \frac{C_2}{[H^+]}$$

for the rate of reactivation should also hold if the assumption is made that the rate of reactivation is directly proportional to the hydroxyl ion concentration. The proportionality constant C_2 has to be replaced then by the equivalent product of two constants, namely,

$$C_2 = cK_w \text{ where } K_w = \text{dissociation constant of water}$$

Hence

$$k_2 = \frac{cK_w}{[H^+]} \tag{7}$$

or

$$pk_2 = pc + pK_w - p\text{H} \tag{8}$$

K_w is known to increase with rise in temperature (Michaelis (1926)). The observed increase in the rate of reactivation with temperature may thus be due only partly to

TABLE II

REACTION: INACTIVE → ACTIVE; $pk_2 = pC_2 - pH$ or $pk_2 = pc + pK_w - pH$

Temperature	5°C.		15°C.		25°C.		35°C.
pC_2	13.06		12.33		11.76		11.30
$C_2 \times 10^{15}$	87		467		1740		5000
Q_{10}		5.37		3.73		2.87	
$K_w \times 10^{15}$	2.72		5.82		12.73		27.0
$c = \frac{C_2}{K_w}$	32.3		80.2		136.8		185.2
Q'_{10} = ratios of c		2.49		1.70		1.35	

the effect of temperature on the intrinsic rate of the reaction, while a great deal of the increase may be due to the increase in the concentration of OH^- ions even though the concentration of H^+ ions is kept constant. Thus, all functions of k_2 relating to changes in temperature, such as

$$Q_{10}, \frac{d\,pk_2}{dT}, \frac{d\,pk_2}{d(1/T)}$$

should be corrected for changes in K_w with temperature.

The corrected values of Q_{10} for reactivation (Q'_{10}) are shown in Table II. The corrected values are of about the same magnitude or less than those for inactivation shown in Table I.

EFFECT OF TEMPERATURE ON THE SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE Mention was made in a preceding section (page 1155) that a solu-

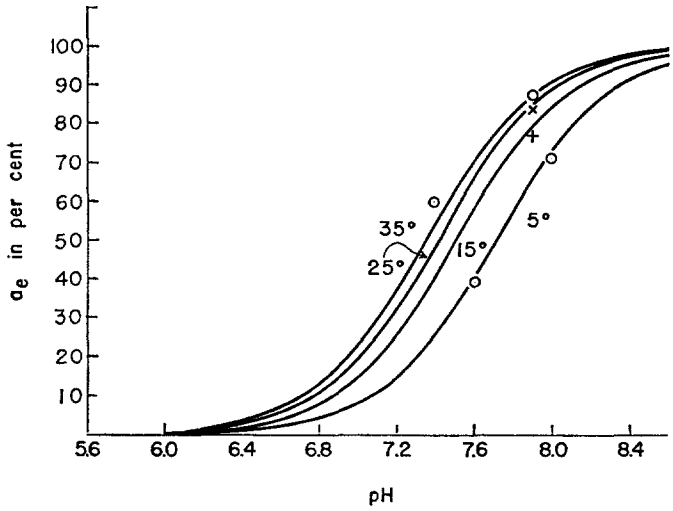

FIGURE 11. Plotted values of a_e *vs.* pH for various temperatures.

tion of activated or partly activated alkaline phosphatase equilibrated at a higher temperature, say at 30 or 35°C., gradually loses part of its activity when cooled and stored at 5°C.; also, *vice versa*, a solution equilibrated at 5°C. will gain activity when heated to higher temperatures. This shift in the equilibrium value a_e towards the active state with rise in temperature is shown graphically in Fig. 11 where isothermal curves for a_e *vs.* pH are given. The values of a_e were calculated with the aid of the standard equation

$$\frac{100 - a_e}{a_e} = \frac{k_1}{k_2} = K_e \tag{9}$$

whence

$$a_e = \frac{100}{1 + K_e} = \frac{100}{1 + \frac{k_1}{k_2}} \tag{10}$$

The plotted curves show that at any pH in the range of 6.0 to 9.0 the value of a_e increases with temperature. This increase of a_e with temperature is due to the fact that k_2 increases with temperature faster than k_1; *i.e.*, due to the greater values of Q_{10} for reactivation over those for inactivation.[3]

THE THERMODYNAMICS OF REVERSIBLE INACTIVATION OF ALKALINE PHOSPHATASE Tables III, IV, and V contain a list of the thermodynamic data

[3] The gradual rise in a_e with temperature may be entirely due to the increase in the concentration of hydroxyl ions with rise in temperature, even as the hydrogen ion concentration is kept constant.

TABLE III

REACTION: ACTIVE → INACTIVE

pH RANGE: 5.6 TO 8.4

Temperature	5°C.	15°C.	25°C.	35°C.	Equations
Intercept pC_1	1.50	1.08	0.66	0.27	See Fig. 9
pk_1 at pH 5.6	4.30	3.88	3.46	3.07	$pk_1 = 0.5\,\text{pH} + pC_1$ Text Equation 3
" " " 8.4	5.70	5.28	4.86	4.47	
$\Delta F_1‡$ at pH 5.6	21,720	21,970	22,184	22,402	$\Delta F_1‡ = 4.58\,T(10318 + \log T + pC_1 + 0.5\,\text{pH})$
" " " 8.4	23,502	23,817	24,095	24,378	Kunitz, 1948, p. 259
E_1 (Arrhenius' constant)		16580			$E_1 = 4.58 \frac{d\,pk_1}{d\left(\frac{1}{T}\right)}$
$\Delta H_1‡$	16,024	16,004	15,984	15,964	$\Delta H_1‡ = E_1 - RT$
$T\Delta S_1‡$, at pH 5.6	−5696	−5967	−6200	−6438	$T\Delta S_1‡, = \Delta H_1‡ - \Delta F_1‡$
" " " 8.4	−7480	−7813	−8110	−8412	
$\Delta S_1‡$ pH 5.6	−20.5	−20.7	−20.8	−20.9	
" " 8.4	−26.9	−27.1	−27.2	−27.3	

TABLE IV

REACTION: INACTIVE → ACTIVE

pH RANGE: 5.6 TO 8.4

Temperature	5°C.	15°C.	25°C.	35°C.	Equations
Intercept pC_2	13.06	12.33	11.70	11.30	See Fig. 10
pk_2 at pH 5.6	7.46	6.73	6.16	5.70	$pk_2 = -\text{pH} + pC_2$
" " " 8.4	4.66	3.93	3.36	2.90	(Text Equation 5)
$\Delta F_2‡$ at pH 5.6	25,734	25,732	25,869	26,118	$\Delta F_2‡ = 4.58\,T(10318 + \log T + pC_2 - \text{pH})$
" " " 8.4	22,170	22,039	22,047	22,167	
E_2 (Arrhenius' constant)		22,900			$E_2 = 4.58 \frac{d\,pk_2}{d\left(\frac{1}{T}\right)}$
$\Delta H_2‡$	22,344	22,324	22,304	22,284	$\Delta H_2‡ = E_2 - RT$
$T\Delta S_2‡$ at pH 5.6	−3,390	−3,408	−3,565	−3,834	$T\Delta S_2‡ = \Delta H_2‡ - \Delta F_2‡$
" " " 8.4	+174	+285	+257	+117	
$\Delta S_2‡$ at pH 5.6	−12.2	−11.8	−12.0	−12.5	
" " " 8.4	+0.62	+1.02	+0.82	+0.32	

TABLE V

REACTION: ACTIVE ⇌ INACTIVE

pH RANGE: 5.6 TO 8.4

Temperature	5°C.	15°C.	25°C.	35°C.	Equations
ΔF at pH 5.6	−4014	−3762	−3685	−3716	$\Delta F = \Delta F_1‡ - \Delta F_2‡$
" " " 8.4	1334	1778	2048	2210	
ΔH		−6320			$\Delta H = \Delta H_1‡ - \Delta H_2‡$
ΔS at pH 5.6	−8.3	−8.5	−8.8	−8.4	$\Delta S = \Delta S_1‡ - \Delta S_2‡$
" " " 8.4	−27.5	−28.1	−28.0	−27.6	

calculated with the aid of standard equations (Kunitz (1948)). All thermodynamic data are given in calories per mole, except $\Delta S‡$ and ΔS which are expressed in calories per degree per mole. The values of pk_1 and pk_2 were obtained by the use of Equations 3 and 5 of this text. The thermodynamic values for the reversible inactivation of chicken intestinal alkaline phosphatase including the values of Q_{10} are of the same magnitude as those of an ordinary chemical reaction and are much smaller than the values observed for denaturation of proteins.

TABLE VI

COMPARISON OF THERMODYNAMIC VALUES FOR REVERSIBLE INACTIVATION OF ALKALINE PHOSPHATASE WITH THOSE FOR REVERSIBLE HEAT DENATURATION OF CRYSTALLINE SOYBEAN TRYPSIN INHIBITOR

	Alkaline phosphatase	Soybean trypsin inhibitor
E_1 (Arrhenius' constant)	16,580	55,000
E_2 " "	22,900	(−1300)
ΔH	−6,320	57,000
$\Delta S_1‡$	−20.5 at pH 5.6; −27 " " 8.4	95
$\Delta S_2‡$	−12 " " 5.6; 0.7 " " 8.4	−84
ΔS	−8.5 " " 5.6; −28 " " 8.4	180

Table VI contains a summary of the thermodynamic values for the reversible inactivation of purified chicken intestinal alkaline phosphatase described in this text, and also, for comparison, the corresponding thermodynamic values for reversible heat denaturation of crystalline soybean trypsin inhibitor (Kunitz (1948)).

II. REACTIVATION BY ZINC IONS

Chicken intestinal alkaline phosphatase inactivated at pH 4.5 is rapidly reactivated on the addition of Zn ions at the same pH. The rate as well as the extent of reactivation by Zn ions increases with increase of pH, temperature, and Zn ion concentration. Of a large number of metal cations investigated, zinc proved to be the only cation capable of antagonizing the inactivation caused by the action of hydrogen ions on the enzyme. Zinc, however, is unable to replace magnesium as a cocatalyst for the enzymatic hydrolysis of organic phosphate by alkaline phosphatase. Zinc ion is also effective in reversing inactivation caused by the chelating reagent versene (disodium ethylenediaminetetraacetic acid). Zinc, however, is not specific in this case; other ions such as Pb, Ca, and La are also able to reactivate to a certain extent the

versene-inactivated enzyme, zinc ion being most effective in that respect. Zinc ion appears to bring about only a slight improvement in the activity of the spontaneously reactivated enzyme at pH 8.5.

Experimental

1. REACTIVATION BY ZINC IONS

Reactivation mixture

1.0 ml. 0.2 M acetate buffer pH 4.5
0.8 ml. of zinc or other salt solutions, or water
0.2 ml. of 0.1 per cent solution of stock-inactivated enzyme in 0.1 M acetate buffer pH 4.5

The mixture was incubated at constant temperature; samples of 0.1 ml. were added at various times to 3.5 ml. of standard assaying mixture for activity measurement.

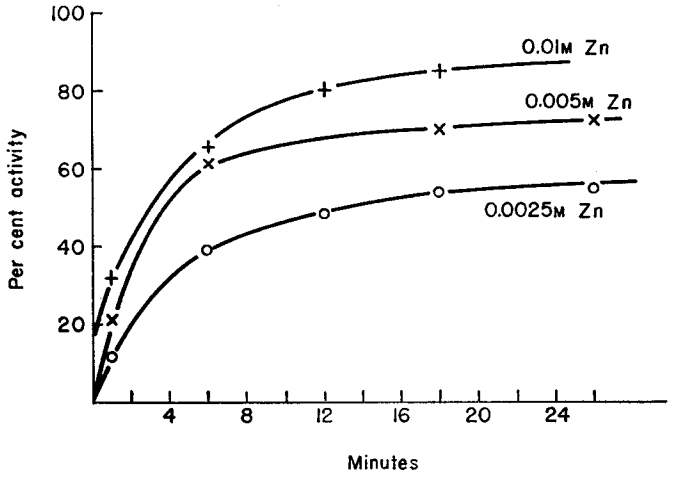

FIGURE 12. Effect of concentration of Zn ions on rate of reactivation of alkaline phosphatase at pH 4.9 and 15°C.

Fig. 12 shows the effect of varying the concentration of $ZnCl_2$ on the rate of reactivation at pH 4.9 and 15°C. of acid-inactivated chicken alkaline phosphatase.

Fig. 13 shows the effect of pH on the rate of reactivation in 0.004 M $ZnCl_2$ while the effect of temperature is shown in Fig. 14. Other cations, such as Mg, Ca, Sr, Ba, Mn, Ni, Co, Cd, Be, Cu, Pb, Fe^{++}, Fe^{+++}, Al, Ce, La, in a wide range of concentrations of their chloride salts, failed to cause any perceptible reactivation of the enzyme at pH 4.5 or 4.9.

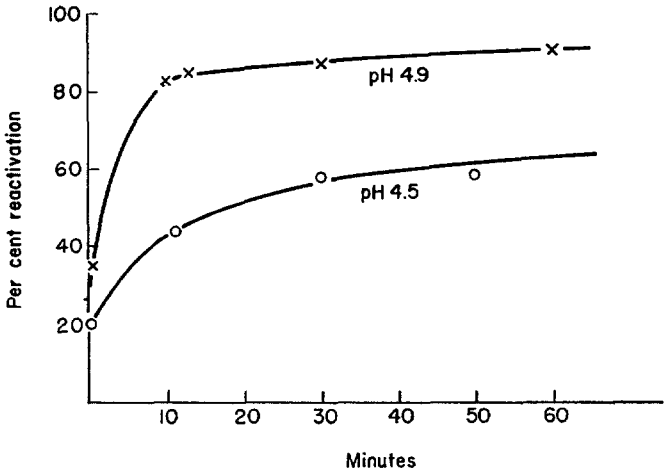

FIGURE 13. Effect of pH on rate of reactivation by Zn.

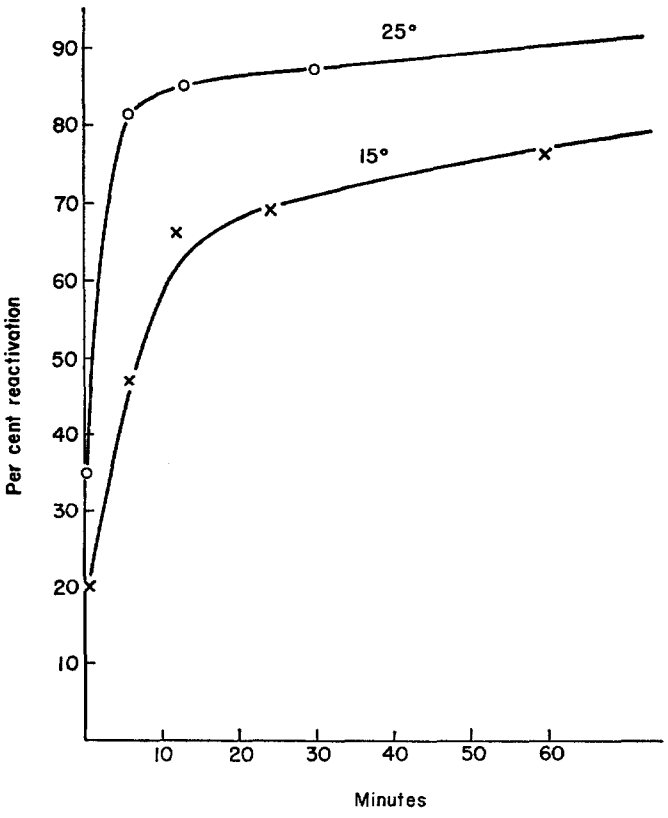

FIGURE 14. Effect of temperature on rate of reactivation by Zn.

2. EFFECT OF VARIOUS METAL CATIONS AS COCATALYSTS IN THE ENZYMATIC HYDROLYSIS OF CARBOXYPHENYL PHOSPHATE BY CHICKEN INTESTINAL ALKALINE PHOSPHATASE

A series of assaying mixtures was made up in which the generally used magnesium salt was replaced by various other metal ions of the same concentration. The effect of the various ions on the rate of enzymatic hydrolysis of the

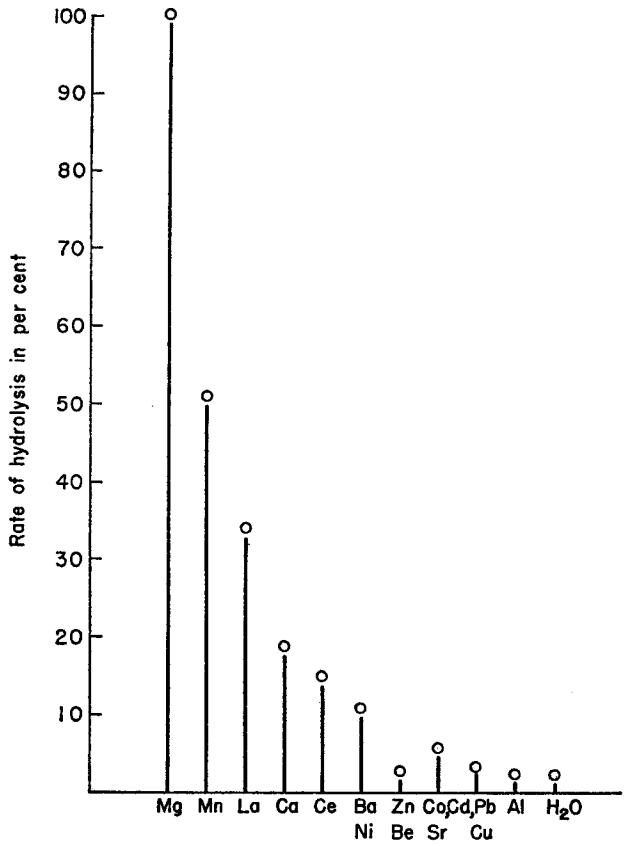

FIGURE 15. Effect of various metal ions on the rate of enzymatic hydrolysis of carboxyphenyl phosphate by chicken intestinal alkaline phosphatase.

standard substrate is shown graphically in Fig. 15. Mg can be partly replaced by Mn and to a lesser extent by La, Ca, Ce, Ba, and Ni, but not by Zn or Al or Be, etc.

3. EFFECT OF METAL IONS ON THE STABILITY OF CHICKEN ALKALINE PHOSPHATASE IN 0.1 M TRIS BUFFER, PH 8.5

Procedure A stock solution of 0.1 per cent of dry inactive enzyme in 0.1 M tris buffer, pH 8.5, was incubated for 1 hour at 35°C. for the enzyme to reach its full

activity. The stock solution of the reactivated enzyme was then stored at about 3°C. Aliquot portions of 4.4 ml. of the stock of reactivated enzyme were mixed with 0.6 ml. of 0.01 M of various metal salts—mostly chlorides—and incubated at 25°C. Samples of 0.1 ml. of the mixtures were then pipetted at various times into 3.5 ml. of standard assaying mixture for activity measurements.

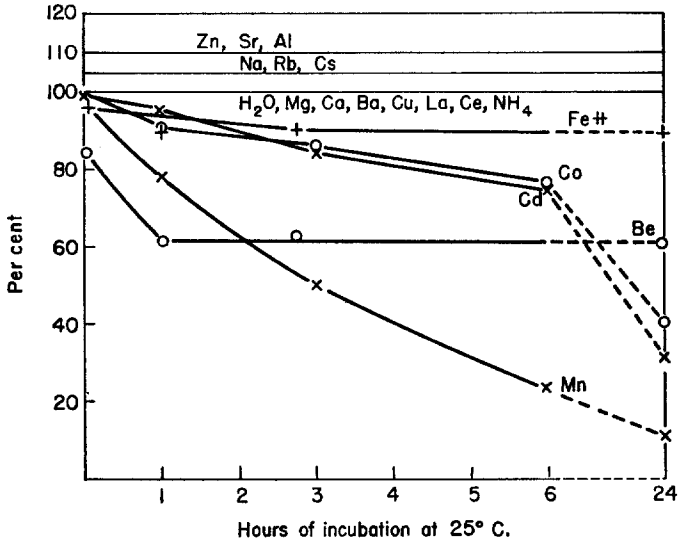

FIGURE 16. Effect of various metal ions on the stability of chicken alkaline phosphatase in 0.1 M tris buffer, pH 8.5.

The results are shown in Fig. 16. Most cations had no appreciable effect on the activity of the enzyme in tris buffer, pH 8.5. Manganese showed a distinct inactivating effect on the enzyme. A depressing effect was also shown by Be, Co, Cd, and Fe^{++} but to a lesser extent than by Mn ions, while Zn and also Sr and Al appeared to have a slight activating effect on the enzyme.

The writer was assisted in this work by Eva Meudt and Theodore Levine.

CONCLUSIONS

The low thermodynamic and kinetic values for the reversible inactivation of chicken intestinal alkaline phosphatase as compared with those for reversible heat denaturation of enzymes and other proteins suggest that the inactivation discussed here is not related to denaturation. This is also confirmed by the fact that the active enzyme loses activity when stored at 5°C. and regains activity when brought back to 25 or 35°C., while in reversible heat denaturation activity is regained as the temperature is lowered. On the other hand, the

specificity of zinc ions as a reactivator even at pH 4.5 and also the recent report by J. C. Mathies (1958) on the detection of the presence of measurable amounts of zinc in purified preparations of kidney phosphatase suggest that the active pure enzyme is possibly a complex of zinc and protein stable at pH 8 to 9 but reversibly dissociable at lower pH, thus bringing about the inactivation of the enzyme. The addition of an excess of Zn ions causes a shift in the Zn-protein equilibrium toward the complex state even at pH 4.5, thus bringing about reactivation.

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