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Acid Inactivation of and Incorporation of Phosphate into Alkaline Phosphatase from *Escherichia coli*

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1. Alkaline phosphatase of *Escherichia coli* undergoes below pH 6·0 a reversible acid inactivation that has been studied and related to the extent of uptake of inorganic phosphate occurring below pH 6·0. 2. The rate of inactivation is rapid in the first few minutes but later it decreases markedly. Temperature, pH, composition of buffer and other factors have an important effect on the inactivation. 3. About 60% of the activity lost at pH values above 3·5 is rapidly recovered when the enzyme is taken back to pH 8·0, independently (within certain limits) of the extent of the inactivation. 4. Phosphate and Zn^{2+}, although very good protectors of the inactivation by acid, are not by themselves able to reverse the acid inactivation. 5. Inorganic phosphate seems not to be incorporated into the acid-inactivated enzyme. 6. Incorporation of more than one mole of phosphate/mole of enzyme has been obtained, but the phosphate residues seem to be incorporated to serine residues with a common sequence, suggesting two identical active serine residues/molecule of active enzyme.

During a study of the behaviour of alkaline phosphatase from *Escherichia coli* on CM-cellulose columns it was observed that the enzyme lost its activity below pH 5·0, but that most of it could be recovered in a time-dependent fashion on incubation at neutral pH values.

Alkaline phosphatase has been shown to incorporate inorganic phosphate (Engström, 1961*a*, 1962; Schwartz & Lipmann, 1961), but, unlike the enzymic activity, which is optimum at alkaline pH values, this incorporation increases as the pH diminishes. Engström (1962) found that the incorporation of [^{32}P]phosphate at pH 4·0 and 5·0 was about 0·9 mole/mole of enzyme (mol.wt. 80000). Schwartz (1963) reported that optimum incorporation occurred at about pH 4·0 (about 0·6 mole/mole of enzyme), but poorly reproducible results were obtained at low pH values. Dependence of the incorporation on temperature was also reported (Engström, 1961*b*), the incorporation being lower at higher temperatures.

It seemed to us of interest that both incorporation of inorganic phosphate and enzyme inactivation take place at about the same pH. It is generally accepted that alkaline phosphatase from *E. coli* consists of two identical sub-units (Rothman & Byrne, 1963). Two atoms of zinc/molecule of active enzyme are tightly bound to the protein (Plocke, Levinthal & Vallee, 1962). Only one sequence containing serine phosphate and accounting for most of the incorporated radioactive phosphate has been found (Milstein, 1963, 1964; Schwartz, Crestfield & Lipmann, 1963). However, as mentioned above, incorporation of inorganic phosphate has usually been less than 1 mole/mole of enzyme.

In the present work we studied the inactivation of alkaline phosphatase from *E. coli* at acid pH and found that phosphate has a very important effect on this process. The incorporation of inorganic phosphate takes place before inactivation, the latter being responsible for the low incorporation values reported previously. By using these findings we have been able to extend the study of phosphate incorporation to lower pH values.

MATERIALS AND METHODS

Enzyme preparation. Alkaline phosphatase was prepared from *E. coli* by the general procedure of Garen & Levinthal (1960) with minor modifications (Milstein, 1964).

DEAE-cellulose columns were used as described by Garen & Levinthal (1960). After being run through the first column (gradient: 0·02–0·2 M-tris buffer, pH 7·4), the enzyme was found not to be pure. Considerable tailing of the activity was also observed, but the enzyme eluted at the head and the tail of the main peak showed the same elution pattern even after mixing and repeating the chromatography, and no separation into different peaks could be detected. In

most of the experiments enzyme that was passed through a second column (Garen & Levinthal, 1960) (gradient: 0·04–0·2 M-NaCl in 0·1 M-tris buffer, pH 8·6) was used. From the value of the turnover number, homogeneity on DEAE-cellulose chromatography and other data we deduce that this preparation was as pure as that described by Garen & Levinthal (1960).

Enzyme activity. The enzyme assays were made by following the changes in the extinction at 410 mμ due to the hydrolysis of *p*-nitrophenyl phosphate. Unless otherwise indicated, 20 μl. of enzyme was added to 3 ml. of 0·1 M-tris buffer, pH 8·0, containing $BaCl_2$ (0·27 M) and *p*-nitrophenyl phosphate (0·2 mg./ml.). Enzyme activity was followed at room temperature (between 20° and 25°) in a recording Beckman DK 2-A or in a Beckman DU spectrophotometer. Activities were expressed as the increase of extinction per minute during the assay (ΔE/min.).

Protein concentration. Protein estimations were made at 280 mμ. A value of $E^{1\%}_{280}$ of 8·0 for the enzyme dissolved in distilled water was found on the basis of dry-weight measurements and was used in this work. A very similar value has been reported by Plocke *et al.* (1962). The E_{260}/E_{280} ratio was 0·73.

The enzyme had a turnover number of 3200 moles of substrate/mole of enzyme (mol. wt. 80000) at about 25° and under the conditions of assay described above; the activation by 0·27 M-Ba^{2+} was about 15% more efficient than that by 1 M-tris.

Kinetics of enzyme inactivation at low pH *values and its reversal at* pH 8·0. The enzyme was dialysed in the cold against 1 mM-tris–HCl buffer, pH 7·4, and when necessary diluted with the same buffer. After the addition of buffer to bring the pH of the enzyme solution to the value at which inactivation was followed, the enzyme was incubated for different times in a bath, keeping the temperature within ±0·5° of the indicated value. The velocity of acid inactivation is strongly dependent on temperature. To obtain reproducible results (especially when the incubation is carried out at 0°) all the reagents were kept in the same bath before mixing until equilibrium was reached. Then 20 μl. of the solution was withdrawn for the assay of enzyme activity at different times. Assays were mostly made in duplicate. A control with the enzyme at the same dilution but at pH 7.2 was always run in parallel and the inactivation was expressed as percentage of enzyme activity lost compared with the control. In general the control was very stable, but some loss of activity was observed on long incubation periods and at higher temperatures. When the activity of the control was less than 85% of its original activity the experiment was discontinued.

To study the reactivation of the inactive enzyme, the solution was diluted with an equal volume of 0·5 M-tris buffer, pH 8·0, and the activity at different times was determined on 20 μl. samples. The recovered activity is expressed as the percentage of the initial activity, before the enzyme was taken to low pH.

When the enzyme inactivated at low pH was assayed at pH 8·0, a slight recovery of activity occurred. The time-course of the reaction during assay was therefore not quite linear but showed a slow increase in *p*-nitrophenyl phosphate hydrolysis per unit time. However, this was very slow and for short times (about 1–2 min.) approximately straight lines were observed in the recording spectrophotometer graphs. This contrasts with the rapid recovery of activity shown in Table 3. The difference may be due to the presence of substrate and products that probably inhibit not only inactivation but also reactivation. Other factors that may affect the reactivation velocity during activity assays are low concentration of protein and high concentration of Ba^{2+}.

Studies on the incorporation of inorganic phosphate. The incorporation of radioactive phosphate under different conditions was made according to the procedure described by Engström (1961*a*, 1962). To compare the extent of inactivation and ability to incorporate inorganic phosphate, a sample of enzyme was dialysed in the cold against 1 mM-tris buffer, pH 7·4. It was then divided into two equal samples: the first (inactivated sample) was adjusted to pH 4·0 with acetate buffer (final concn. 0·2 M) and the second (control sample) was diluted to the same volume with 1 mM-tris buffer, pH 7·4. Both were kept for 2 hr. at 28° and their enzymic activities were determined. All the reagents were incubated in an ice bath before incubation with [^{32}P]phosphate. The [^{32}P]phosphate was then added to both, and acetate buffer (final concn. 0·2 M) to the control to adjust its pH to 4·0. After 10 min. in the ice bath the reaction was stopped with 2 N-HCl, and the enzyme was precipitated with acid–acetone and washed as described by Engström (1962); however, about 10% of removable phosphate was still present after three washings, and so five washings were sometimes used. The precipitated enzyme was dried and dissolved in 67% (v/v) acetic acid. Samples were taken for radioactive assay and for electrophoresis at pH 3·5 to correct for unbound phosphate. Partial acid hydrolysis was made with 5·7 N-HCl at 100° for 30 min. and ionophoresis was carried out at pH 3·5 as described by Milstein & Sanger (1961). In Fig. 4, owing to small changes of the pH of the buffer in the ionophoresis tanks, some differences in the pattern of radioactive peptides were observed (see Milstein, 1964). Radioactivity measurements were carried out in a Nuclear–Chicago Corp. gas-flow automatic counter.

RESULTS

Inactivation of alkaline phosphatase from Escherichia coli *at low* pH *values.* When alkaline phosphatase was incubated at acid pH values the activity was slowly lost at a rate depending on the pH of the incubation mixture. Fig. 1 shows the inactivation observed at pH 5·5, 5·0, 4·0 and 3·0. Above pH 5·0 the inactivation was too slow to be measured with any accuracy, and in fact none was observed above pH 6·5. After the initial period of rapid inactivation, the rate decreased markedly. The proportion of original activity lost in the initial rapid phase increased with decreasing pH. The activity of the controls at pH 7·2 remained within 10% of its original value.

The nature of the buffer was very important in the inactivation process. Citrate buffer, for instance, was more effective than acetate, and at pH 3·0 the inactivation in citrate at 0° was very similar to the inactivation in acetate at 28°, and much faster than the inactivation at pH 2·7 in potassium chloride–hydrochloric acid buffer at 0°:

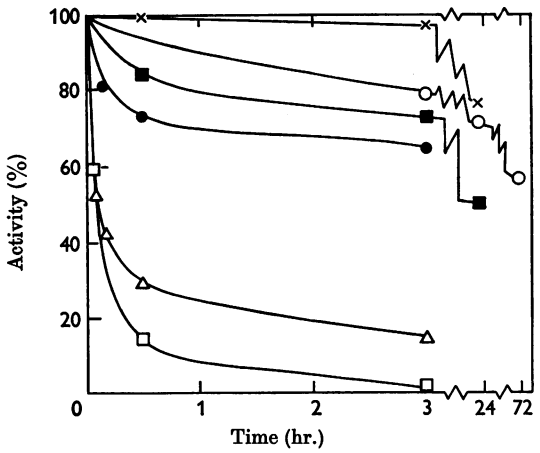

Fig. 1. Inactivation of alkaline phosphatase at acid pH values. The enzyme was incubated in 0·2 M-acetate buffer at the pH specified and at different temperatures. Samples (20 μl.) were assayed for activity at different times. ×, pH 5·5 and 28°; ○, pH 5·0 and 0°; ■, pH 5·0 and 15°; ●, pH 5·0 and 28°; △, pH 4·0 and 28°; □, pH 3·0 and 28°.

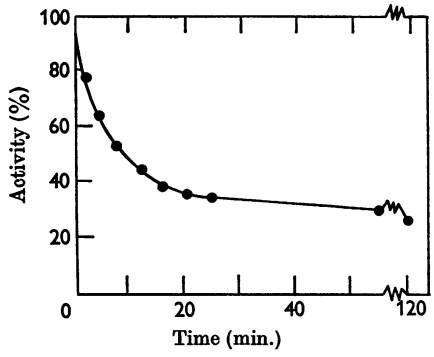

Fig. 2. Inactivation of alkaline phosphatase at pH 4·0. The enzyme (43 μg./ml.) was incubated at 28° in 0·2 M-acetate buffer, pH 4·0. Samples (20 μl.) were withdrawn and assayed. Activity is plotted as percentage of a control run in parallel at pH 7·0. Initial activity was 0·196 ΔE/min. Activity of the control after 2 hr. was 0·195 ΔE/min.

in one experiment, for instance, with 0·25 M-citrate buffer, pH 3·0, 98% inactivation was observed in 1 hr., whereas only 32% inactivation was observed with 0·2 M-potassium chloride–hydrochloric acid buffer, pH 2·7 (both incubations at 0°).

The inactivation that takes place in the first 120 min. was followed in some detail and the curve obtained at pH 4·0 is shown in Fig. 2. After the initial rapid rate, inactivation continued at a lower rate. After much longer periods the measurements were inaccurate because of instability of the control.

Table 1. *Effect of metal ions on the acid inactivation of alkaline phosphatase*

Incubations were carried out in 0·2 M-acetate buffer, pH 5·0, at 28°. The protein concn. was 42 μg./ml. in Expt. A and 190 μg./ml. in Expt. B. 100% activity in Expt. A was 0·190 ΔE/min. and in Expt. B was 0·880 ΔE/min. —, Not determined.

	Added salt (final concn.)	Inactivation (%) after incubation for: 10 min.	3 hr.	6 hr.	9 hr.	24 hr.
Expt. A	None	—	25	—	36	—
	$ZnCl_2$ (10 μM)	—	<5	—	14	—
	$ZnCl_2$ (1 mM)	—	<5	—	<5	—
	$MgCl_2$ (10 μM)	—	21	—	28	—
	$MgCl_2$ (1 mM)	—	7	—	21	—
	$CoCl_2$ (10 μM)	—	24	—	—	—
	$CoCl_2$ (1 mM)	—	44	—	54	—
Expt. B	None	15	—	35	—	53
	$ZnCl_2$ (1 μM)	17	32	35	—	53
	$ZnCl_2$ (10 μM)	<5	17	17	—	—
	$ZnCl_2$ (0·1 mM)	<5	<5	<5	—	13
	$ZnCl_2$ (10 mM)	<5	<5	<5	—	8

The inactivation depended on temperature and on concentration of buffer. Higher concentration of buffer increased the rate of inactivation at low pH values. For instance, after 3 hr. at pH 4·0 (at 20°) values of inactivation were 60 and 81% respectively in 0·01 M- and 0·2 M-acetate buffer. The inactivation after 3 hr. at 28° was 85% in 0·2 M-acetate buffer, pH 4·0, compared with the value of 20% obtained with 0·01 M-acetate buffer, pH 3·5.

The enzyme was protected by Zn^{2+} from the acid inactivation. Some protection was also obtained with Mg^{2+} (1 mM) but not with Co^{2+} (10 μM and 1 mM) (Table 1). At the higher concentration (1 mM), however, Co^{2+} seemed to increase the inactivation at pH 5·0. The effect of Zn^{2+} was to lower the maximum inactivation.

A similar protective effect was observed when the incubations were made in the presence of phosphate (Table 2). The incubations in the experiments of Table 2 were carried out at 0° for two reasons: first, the rate of the inactivation is lower and the kinetics are easier to follow at this temperature; secondly, phosphate-incorporation studies (see below) were made at this temperature and direct comparison of the results was of interest. The results indicate that during phosphate incorporation at low pH both inactivation and protection take place, and, since the temperature also is such an important factor (Fig. 1), phosphate-incorporation studies require careful control of the above variables for reproducibility.

Table 2. *Effect of phosphate on the acid inactivation of alkaline phosphatase*

Incubations were carried out in 0·2 M-acetate buffer, pH 4·0 (Expt. A), 0·25 M-citrate buffer, pH 3·0 (Expt. B), or 0·2 M-KCl–HCl buffer, pH 2·7 (Expt. C) at 0°. 100% activity (ΔE/min.) was 0·350 in Expt. A; 0·126 in Expt. B and 0·100 in Expt. C. The enzyme concn. (μg./ml.) was 77 in Expt. A, 27 in Expt. B and 22 in Expt. C. The percentage inactivation is indicated in parentheses. —, Not determined.

	Concn. of phosphate (μM)	Enzyme:phosphate molar ratio	Activity (ΔE/min.) after incubation for: 2 min.	6 min.	10 min.	30 min.	1 hr.	1·5 hr.
Expt. A	0	—	0·340 (<5)	0·330 (5·5)	0·300 (14)	0·265 (24)	0·235 (33)	0·170 (51·5)
	9·6	1:10	0·355 (<5)	—	0·328 (6)	0·315 (10)	0·298 (15)	—
	96	1:100	0·360 (<5)	0·350 (<5)	0·340 (<5)	0·350 (<5)	0·360 (<5)	—
Expt. B	0	—	—	0·072 (43)	0·052 (58)	0·010 (92)	—	—
	34	1:100	—	0·088 (30)	0·078 (38)	0·042 (67)	—	—
Expt. C	0	—	—	0·098 (<5)	0·080 (20)	—	0·068 (32)	0·050 (50)
	28	1:100	—	0·098 (<5)	0·110 (<5)	—	0·098 (<5)	

Table 3. *Reversibility at* pH 8·0 *of the acid inactivation of alkaline phosphatase*

Inactivation was carried out in 0·2 M-acetate buffer. Reactivation was followed after the addition of 1 vol. of 0·5 M-tris buffer, pH 8·0. Values in parentheses indicate the percentages of lost activity recovered.

Expt. no.	Conditions of inactivation: pH	Temp.	Time (hr.)	Inactivation (%)	Final activity (% of original) after reactivation for: 5 min.	10 min.	30 min.	3 hr.
1	5·0	0°	72	45	—	82 (60)	—	78
2	4·0	0	72	61	—	73 (56)	—	73
3	3·5	28	3	89*	67 (63)	—	62	—
4	3·0	28	3	98	25 (23)	—	—	—
5	4·0	28	0·083	46	—	—	80 (57)	—
6	4·0	28	0·75	77	—	—	72 (63)	—
7	4·0	28	2	85	—	—	68 (62)	—
8	4·0	28	5	88	—	—	63 (58)	—

* Concn. of acetate buffer was 0·01 M.

Reversal of the acid inactivation of alkaline phosphatase. When alkaline phosphatase, inactivated at acid pH values, was incubated at pH 8·0, part of the lost activity was recovered. The final activity depended on the amount of inactivation, but the proportion of lost activity recovered did not (Table 3). Under the conditions of Table 3, and except when inhibition was performed at pH 3·0 (Expt. 4), about 60% of the lost activity was recovered in almost all experiments. Expts. 5–8 show that the recovery of the activity lost on incubation at pH 4·0 for up to 5 hr. was independent of the time of inactivation at acid pH. No significant difference in the amount of activity recovered was observed when inactivation was carried out at various pH values except when extreme conditions were used, as in Expt. 4. In this case a much lower reactivation was obtained.

As seen above, Zn^{2+} and phosphate had a striking effect on the inactivation by acid. However, when added after the fast inactivation was completed, neither of them significantly affected the equilibrium. No reversibility comparable with that obtained at pH 8·0 was obtained with either Zn^{2+} or phosphate (Table 4), although a slight increase in activity, perhaps within the experimental error, was observed in the presence of Zn^{2+}.

Incorporation of phosphate and acid inactivation. One problem that arises is whether the incorporation of phosphate takes place only in the active enzyme or if the inactive form is also capable of such incorporation. A sample of enzyme was therefore submitted to acid inactivation and a duplicate was used as a control; incorporation of [^{32}P]-phosphate was then studied. As shown in Table 5 incorporation into the inactivated enzyme was much lower. The values of the inactivation and of the incorporation were in good agreement in Expt. B but not so good in Expt. A. The incorporation in both controls of Table 5 varied considerably. This is not surprising since, as discussed above, phosphate protects the enzyme against acid denaturation; in Expt. B the phosphate:enzyme molar ratio during incubation was 60, which as shown above gives a better protection than the lower ratio (9) used in Expt. A. For this reason we give more weight to Expt. B. The labelling in both inactivated and control enzyme mixtures in Expt. A seemed to occur at the same serine residue, since the same pattern of radioactive peptides was obtained after ionophoresis and radioautography of the partial acid hydrolysates of the labelled proteins (Fig. 3).

Incorporation of inorganic phosphate could be

Table 4. *Inability of* Zn^{2+} *and phosphate to reverse the acid inactivation of alkaline phosphatase*

Inactivations were carried out in 0·2 M-acetate buffer, pH 5·0 (Expt. A), or pH 4·0 (Expt. B), for 72 hr. at 0° (Expt. A) or 3 hr. at 28° (Expt. B). The inactivated enzyme (50% inactive in Expt. A and 86% inactive in Expt. B) was then (zero time) diluted with an equal volume of a suitable solution of NaH_2PO_4 or $ZnCl_2$ to give the final concentrations indicated and a protein concentration of 150 μg./ml. (Expt. A) or 76 μg./ml. (Expt. B). Samples were assayed after incubation at 28° (Expt. A) or 0° (Expt. B) for different times. The percentages of the initial activity are indicated in parentheses. Initial activity was 0·670 ΔE/min. in Expt. A and 0·346 ΔE/min. in Expt. B.

Added salt (final concn.)		Activity (ΔE/min.) after incubation for: 5 min.	1 hr.	3 hr.
Expt. A	None	0·337 (50)	0·300 (45)	0·325 (49)
	$ZnCl_2$ (10 μM)	0·387 (57)	0·375 (56)	—
	$ZnCl_2$ (0·1 mM)	0·392 (58)	0·337 (50)	0·362 (54)
	$ZnCl_2$ (10 mM)	0·362 (54)	0·362 (54)	0·362 (54)
Expt. B	None	0·046 (13)	0·050 (14)	—
	NaH_2PO_4 (48 mμM)*	0·042 (12)	0·046 (13)	—

* Enzyme/phosphate molar ratio was 1:100.

Table 5. *Incorporation of inorganic* [^{32}P]*phosphate by acid-inactivated alkaline phosphatase*

Acid inactivation was carried out in 0·2 M-acetate buffer, pH 4·0, for 2 hr. Then 6·9 mμmoles of enzyme and 60 mμmoles of [^{32}P]phosphate containing 60×10^4 counts/min./mμmole (Expt. A) or 8·7 mμmoles of enzyme and 516 mμmoles of [^{32}P]phosphate containing 8×10^4 counts/min./mμmole (Expt. B) were incubated at 0° and pH 4·0 for 10 min. Incorporation was measured by the radioactivity associated with the protein after precipitation as indicated in the Methods section. The values for ^{32}P incorporation (column 4) have been corrected for free phosphate found after ionophoresis at pH 3·5 of the precipitated and washed enzyme. Free phosphate (percentage of total) was 4% (control) and 13% (acid-inactivated) in Expt. A; in Expt. B the values were 0·1 and 0·4% respectively. Five washings were performed on the precipitated enzyme in Expt. B.

	Enzyme	Phosphate:enzyme molar ratio during incubation	$10^{-4} \times$ ^{32}P incorporation (counts/min./mμmole of enzyme)	Phosphate incorporation (moles/mole of enzyme)
Expt. A	Control	8·8	49	0·82
	77% inactive	8·8	17	0·28
Expt. B	Control	60	8·3	1·01
	70% inactive	60	2·5	0·30

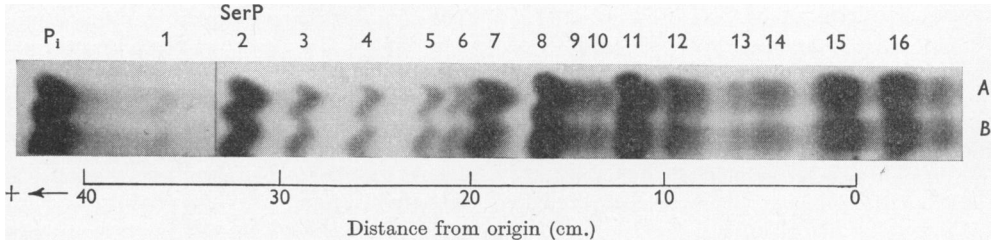

Fig. 3. Radioautograph of ionogram (at pH 3·5 and 35 v/cm. for 2 hr. on Whatman 3MM paper) of partial hydrolysates (in 5·7 N-HCl for 30 min. at 100°) of ^{32}P-labelled alkaline phosphatase: *A*, inactivated with acid before labelling; *B*, control. The samples of enzyme used were fractions from Expt. A of Table 5. The numbers refer to the bands described by Milstein (1964). SerP, Serine phosphate.

improved at lower pH values provided that care was taken to avoid acid denaturation. By using a suitable concentration of phosphate, added before the enzyme was taken to acid pH and keeping all the reagents at 0°, the incorporation increased even at pH 3·0, when a value of 1·3 moles of phosphate/mole of enzyme was obtained (Table 6).

The increase in the incorporation of [^{32}P]phosphate at lower pH does not seem to be due to the presence of a second phosphorylating site. Partial acid hydrolysates of proteins which were labelled at pH 5·0 and 2·7 showed the same pattern of radioactive peptides (Fig. 4). Moreover, Table 6 shows that the increase in radioactivity could be accounted for by the increase in the yield of the radioactive bands that are characteristic of the reactive serine in the sequence (Ser or Thr)-Asp-Ser-Ala-neutral residue (Milstein, 1963, 1964).

Table 6. *Incorporation of* [^{32}P]*phosphate at low* pH *values into alkaline phosphatase protected from acid inactivation*

Incorporation of [^{32}P]phosphate was carried out in 1 ml. of 0·1 M-acetate buffer, pH 4·0 and 3·5, and 0·2 M-KCl–HCl, pH 3·0, at 0° for 10 min.; 8·7 mμmoles of enzyme and 516 mμmoles of [^{32}P]phosphate containing 8×10^4 counts/min./mμmole were used. Samples of equal amounts of the labelled enzymes were partially acid-hydrolysed and subjected to paper ionophoresis (same conditions as in Fig. 3). Areas including bands 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15 and 16 were cut out and their radioactivities measured. The precipitated proteins were washed five times and no [^{32}P]-phosphate was detected in the ionograms of the labelled protein. The peptides included in column 4 account for about 90% of the radioactive peptides of the partial acid hydrolysate of ^{32}P-labelled phosphatase from *E. coli* (Milstein, 1964).

	Phosphate incorporation		Total radio-activity in the main peptide bands (counts/min.)	Labelling of main peptide bands (arbitrary units)
pH	(counts/min./mμmole of enzyme)	(moles/mole of enzyme		
4·0	83 000	1·01	18 600	1·00
3·5	100 000	1·12	21 200	1·15
3·0	116 000	1·30	24 500	1·32

DISCUSSION

A kinetic analysis of the acid inactivation of the *E. coli* alkaline phosphatase has been made in Fig. 5. The plot of the logarithm of the percentage of residual activity against time is non-linear. However, the last part of the curve is linear, and if this is extrapolated to zero time and the ordinates of the line subtracted from the corresponding experimental values, points are obtained that lie on a straight line. Such analysis has previously been applied to the digestion of myosin (Mihalyi & Harrington, 1959) and to the photo-oxidation of phosphoglucomutase (Ray & Koshland, 1962), and the results fit a model of two independent first-order reactions.

In our case, the fast reaction accounts for nearly all the inactivation taking place during the first 20 min. of incubation under the conditions of Fig. 2. The slow reaction at this pH and temperature is of little significance even after 2 hr. The results of Fig. 1 indicate that the extent of the fast reaction varies with pH. At pH 4·0 and 28° the inactivation due to the fast reaction is about 65% (see Fig. 5). The question whether at this stage all the enzyme molecules have lost 65% of their activity or whether 65% of the enzyme molecules have lost 100% of their activity has been investigated by studying the phosphorylation of the partial inactivated enzyme. Kinetically these two possiblities cannot be distinguished by the method employed. In both cases a plot of time against the logarithm of the percentage of residual activity (which would eventually be lost during the fast reaction) should give a straight line. In both cases the equation:

$$X = e^{-Kt}$$

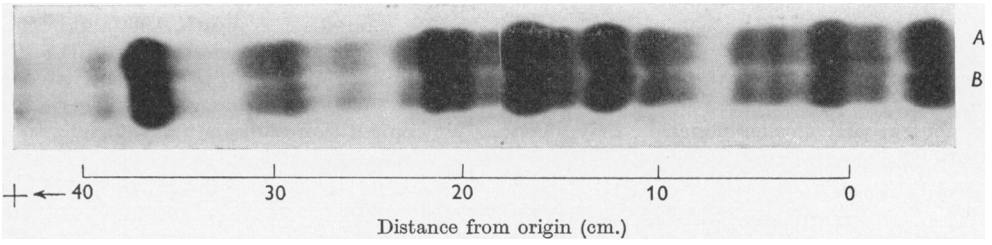

Fig. 4. Radioautograph of ionogram (at pH 3·7 and 60 v/cm. for 1·5 hr. on Whatman 3MM paper, with P_i run out of the paper) of partial acid hydrolysates of ^{32}P-labelled alkaline phosphatase: *A*, at pH 5·0; *B* at pH 2·7. Other labelling conditions were similar to those of Table 6.

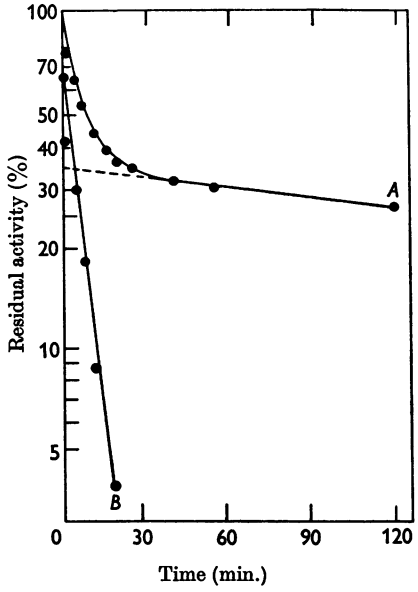

Fig. 5. Kinetics of acid inactivation. The data used were from Fig. 2. *A*, Slow inactivation; *B*, fast inactivation.

applies, X_t being the percentage of residual 'acid-labile' activity at time t and K a constant whose meaning differs in each case. That phosphorylation and enzyme activity are closely related has been shown previously (Engström, 1961*a*; Schwartz, & Lipmann, 1961), and, since the incorporation of phosphate is an 'all-or-none' reaction, it should be possible to distinguish between a situation where all molecules are active at a lower rate (complete phosphorylation expected) or where a fraction is inactive (partial incorporation expected). As indicated by the results of Table 5 the acid-inactivated protein seems to be unable to incorporate inorganic phosphate. This provides further evidence that the phosphorylation occurs at the active site. These results therefore suggest that the fast inactivation is due to an equilibrium of the type:

$$E(\text{active}) \overset{H^+}{\rightleftharpoons} E(\text{inactive})$$

However, since only 60% of the activity lost in acid was recovered when the enzyme was brought back to neutral pH, this explanation may be a simplification of a more complex event.

The protection by phosphate suggests that the acid inactivation of the phosphorylated enzyme follows a completely different pattern. A specific effect different from the phospho-enzyme formation and added to it is not excluded, however. An alternative explanation involving an unspecific effect of phosphate seems unlikely, since the rate of inactivations increases with increase of acetate concentration and when citrate buffer is used instead of acetate.

These results could be explained by a general change of the tertiary structure of the protein initiated by the change in the ionic equilibrium of the peptide chains at lower pH values. The phospho-enzyme might have a more stable structure and more extensive changes might be required for the general breakdown of its configuration. An alternative explanation could be that a specific group available in the enzyme but unavailable in the phosphorylated form is responsible for this inactivation. The pK of such a group would lie between 4·0 and 5·0, close to the pK of a carboxyl group from a side chain of a glutamic acid or an aspartic acid residue (which is about 4·0 and 5·0). The pK_2 of phosphate is 5·6 and that of phosphoserine peptides between 5·4 and 6·0 (Fölsch & Österberg, 1959). The pK of a histidine residue in a protein is generally considered to be higher than 6·0 (but see Tanford, 1962). On the other hand, the incorporation of inorganic phosphate into the protein steadily increases between pH 6·0 and 4·0 (see also Schwartz, 1963). There is still a measurable increase in phosphate incorporation down to pH 3·0, but the observed values may not represent maximum incorporation at those pH values and at the phosphate concentration used since the incorporation of inorganic phosphate depends on the concentration of phosphate and on pH. No accurate estimates of the pK of group(s) involved in the incorporation can therefore be made with the data available. However, both phosphorylation and inactivation occur in a similar pH range. An aspartic acid residue is located next to the phosphorylated serine in alkaline phosphatase from *E. coli* (Milstein, 1963; Schwartz *et al.* 1963) and from *Serratia marcescens*, and probably in that from intestine (Milstein, 1964; Zwaig & Milstein, 1964).

Acid inactivation is prevented when Zn^{2+} is present, and that suggests at first a competition with this metal by H^+. It has been shown that Zn^{2+} loses its ability to bind to alcohol dehydrogenase (Vallee & Hoch, 1957), carboxypeptidase (Vallee, Rupley, Coombs & Neurath, 1960) and other zinc-containing metalloenzymes at about pH 5. The fact that the addition of Zn^{2+} to the inactivated enzyme does not reverse the effect of H^+ suggests, however, that competition alone is not sufficient to explain these effects. Further, a decrease in the rate of inactivation is brought about (and more efficiently) by phosphate also. In addition, Co^{2+}, which can partially replace Zn^{2+} in the active enzyme (Plocke & Vallee, 1962), enhances the rate of inactivation by acid. A rather similar complication is observed in the reversibility by various metal ions of *o*-phen-

anthroline inhibition (Plocke & Vallee, 1962). An explanation of these results probably involves changes of the native configuration of the protein, stabilized by Zn^{2+} and phosphate. That phosphate and Mg^{2+} are able to protect the native enzyme against heat denaturation has been shown by Garen & Levinthal, 1960. Participation of the tertiary structure is also suggested by the fact that the inactivation is a time-dependent process and that temperature has a marked effect on it.

Schlesinger & Levinthal (1963) report the effect of pH on the formation of active enzyme from monomers prepared by acid treatment of an inactive phosphatase-like protein obtained from mutants of *E. coli*. Although different conditions and use of a strain with full phosphatase activity are essential differences between the reactivation described in the present paper and that described by Schlesinger & Levinthal (1963), in their study reactivation was a slow process depending on the addition of Zn^{2+}, whereas in the present study 60% of the inactive protein was reactivated in a very short time and without the addition of metal ion. The production of monomers may possibly represent a more drastic effect of the acid treatment than the changes studied in the present paper. The addition of salts during reactivation had a very complicated effect, and in unpublished experiments we have observed reactivations of up to 150% of the original activity.

An amino acid sequence around a unique serine phosphate residue has been described in ^{32}P-labelled alkaline phosphatase (Milstein, 1963, 1964; Schwartz *et al.* 1963). The fact that significantly more than 1 mole of phosphate/mole of protein was incorporated into phosphatase in some of the experiments described above indicates that the molecule contains more than one active site. Incorporation of more than one mole/mole gives a higher labelling of the radioactive bands, described by Milstein (1964), obtained after ionophoresis at pH 3·5 of the partial acid hydrolysate of the ^{32}P-labelled protein, strongly suggesting that a single serine residue is the site of this incorporation. It has been suggested that alkaline phosphatase from *E. coli* consists of two identical sub-units (Rothman & Byrne, 1963); the present findings are in agreement with this suggestion, and indicate further that both chains of the native enzyme are able to incorporate inorganic phosphate.

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