was obtained. One final preparation was homogeneous in the ultracentrifuge.

2. The purified enzyme usually had a high activity and was not activated by Fe^{2+} ions and cysteine. Under certain conditions preparations of lower specific activity were obtained which could be activated to some extent.

3. The amount of iron in the enzyme is considered to be too small to be directly concerned in the action of the enzyme.

4. Some aspects of the kinetics of the enzyme have been studied and discussed.

I thank Dr J. S. D. Bacon for his interest and advice throughout this work, Dr J. J. Connell and Dr I. Mackie of the Torry Research Station (D.S.I.R.) for carrying out the ultracentrifuge runs, Dr R. O. Scott of the Spectrochemistry Department of this Institute for the analyses of metals, and Miss E. Cusens and Miss E. D. Davidson for technical assistance.

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The Amino Acid Sequence Around the Reactive Serine Residue in Alkaline Phosphatase from *Escherichia coli*

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(*Received* 3 *December* 1963)

Study of the active centres of enzymes by direct determination of the amino acid sequence around a labelled amino acid appears to be an important step in elucidation of the mechanism of enzyme action. The first successful attempts were made on proteolytic enzymes by using specific organophosphorus inhibitors. A second approach was to label the enzyme with groups that normally participated in the enzyme reaction. The first such group to be considered was the phosphate in phosphoglucomutase (Anderson & Jollès, 1957). The primary step of specific labelling to study active centres by this technique is being extended to an increasing number of enzymes (Engström, 1961*a*, *b*; Park, Meriwether, Clodfelder & Cunningham, 1961; Grazi, Rowley, Cheng, Tchola & Horecker, 1962). Characterization of the amino acids around the reactive group by isotopic techniques has been used successfully in some cases (Naughton, Sanger, Hartley & Shaw, 1960; Milstein & Sanger, 1961). These techniques have great advantages over the conventional methods for the determination of protein sequences when applied to the determination of short sequences around the active centres, particularly for their comparison in several enzymes. The comparative technique, which showed elastase (Naughton *et al.* 1960), but not phosphoglucomutase (Milstein & Sanger, 1960), to contain the sequence Gly-Asp-Ser-Gly, is applicable to small amounts of enzyme (about 1 mg.) which need not be pure (Milstein, 1961). This technique has now been applied to alkaline phosphatase.

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Alkaline phosphatase from calf intestinal mucosa incorporates inorganic phosphate, and serine phosphate has been identified in partial acid hydrolysates of the ^{32}P-labelled enzyme (Engström, 1961*a*). The fact that the incorporation of [^{32}P]-phosphate also takes place when the substrate glucose 6-[^{32}P]phosphate is used, combined with other studies (Engström, 1962*b*), strongly indicates that the reactive serine residue is part of the active centre. Alkaline phosphatase of *Escherichia coli* also incorporates phosphate in a similar way (Schwartz & Lipmann, 1961; Engström, 1962*a*), and hence it is possible to compare the amino acid sequence around the active serine residue of this enzyme from several mutant strains of the bacteria. The present paper describes the ionophoretic characteristics of the main radioactive peptides obtained on partial acid hydrolysis of the ^{32}P-labelled alkaline phosphatase of *E. coli*. A preliminary report of part of this work has been published (Milstein, 1963).

METHODS

Materials. Non-radioactive serine phosphate peptides were a kind gift from G. Fölsch. The ^{32}P-labelled ovalbumin peptides and di-isopropoxy[^{32}P]phosphinyl-chymotrypsin and -subtilisin were kindly supplied by F. Sanger and D. C. Shaw.

Alkaline phosphatase from Escherichia coli. Alkaline phosphatase from *E. coli* strain K_{12}, Hfr was prepared by the procedure described by Garen & Levinthal (1960), with minor modifications in the technique for growing the bacteria. The composition of the growth medium was the same except that the tris and the sodium glycerophosphate were omitted. It was adjusted to about pH 8 with $NaHCO_3$ (final concn. 0·01 M) and the cells were allowed to grow for 6–8 hr. until the phosphate contaminating the medium was exhausted. A 500 ml. solution of 1 M-$NaHCO_3$, pH 8·2, containing 1 g. of sodium glycerophosphate, was added under sterile conditions from a separating funnel to 20 l. of medium and the flow rate adjusted to complete the addition of the glycerophosphate in about 18 hr. The cells were harvested when the stationary phase was reached. In this way maximum yield of cells under permanent phosphate starvation was achieved. This is necessary for maximum production of the enzyme.

Preparation of ^{32}P-labelled phosphatase. Several procedures were attempted for the preparation of ^{32}P-labelled phosphatase. That most generally used was similar to the one described by Engström (1961*a*). About 2 mg. of enzyme was incubated at 0° for 5 min. with about 10 μ-moles of phosphate containing 0·5–1 mc of ^{32}P, in the presence of 0·1 M-acetate buffer, pH 5·0. The final volume was 1·8 ml. The reaction was stopped by the addition of 0·3 ml. of 3 N-HCl and the protein was precipitated with 8 ml. of acetone. The supernatant was discarded and the precipitate washed twice with acid acetone (8 vol. of acetone plus 2 vol. of 0·5 N-HCl). Finally it was dried *in vacuo* over KOH. In some experiments the labelled protein was precipitated with 10% (w/v) trichloroacetic acid, washed twice with the same solution and then dialysed against large volumes of distilled water.

Enzymic hydrolysis of ^{32}P-labelled phosphatase. Samples of the dry protein were oxidized with performic acid (Hirs, 1956) and then hydrolysed with proteolytic enzymes. The most satisfactory digestion was performed with pepsin (3 × crystallized; Nutritional Biochemicals Corp.). Digestion was performed in a small test tube containing about 1 mg. of protein in 0·6 ml. of 0·01 N-HCl and 400 μg. of pepsin. The tube was kept for 20 hr. at 37° unless otherwise stated.

Partial acid hydrolysis. Several conditions were used. The reaction was always carried out in small test tubes stoppered with a glass bulb with approx. 0·2 ml. of 5·7 N-HCl at 100° for 30 min. unless otherwise indicated. Hydrolysis in 12 N-HCl at 37° for various times was the most frequent alternative. Hydrolysis with dilute acid was with 0·2 ml. of either 0·033 N-HCl (pH 1·5) or 10% (w/v) acetic acid at 105° for various times.

Ionophoretic fractionation of peptides. The peptides were fractionated by high-voltage paper ionophoresis as described by Michl (1951), with the petroleum fraction Varsol (Esso) as a coolant. The buffers and the coloured markers used to standardize the conditions of each run and the positions of the peptide spots were as described by Milstein & Sanger (1961).

Two-dimensional ionophoresis was used as described by Milstein & Sanger (1961). For the comparison of peptides in more than one system the following method was used. The preparations were placed side by side on a sheet of paper (preferably Whatman no. 52, or 3MM for larger amounts of material) in 2-cm.-wide bands. After the first run the paper was radioautographed, and the strips of paper containing the matching peptides were cut out and sewn with a sewing machine on a second sheet of the same paper, and the ionophoresis was carried out at a different pH.

Radioactivity measurements. An automatic gas-flow-type counter (model C110B; Nuclear–Chicago Corp.) was used. When the radioactivity of the ionogram spots was determined, a recording scanner (C100B Actigraph II; Nuclear–Chicago Corp.) was sometimes used. Usually the spots were cut out and counted, or eluted and samples counted on planchets.

RESULTS

According to Engström (1961*a*) and to Schwartz & Lipmann (1961), the labelling of phosphatase with [^{32}P]phosphate is much more efficient under acid than under neutral or alkaline conditions. This was confirmed by experiments in which the labelling was done at pH 5·0 and at pH 7·4. Both preparations gave the same pattern of radioactive peptides when subjected to digestion with trypsin followed by partial acid hydrolysis, indicating that the labelling occurs at the same site. Lower pH values give an even more efficient labelling, provided that the enzyme is protected from the reversible acid denaturation which becomes significant below pH 5·0 (Pigretti & Milstein, 1964).

Maximum specific radioactivity of the labelled residue is desirable for a study of the amino acid

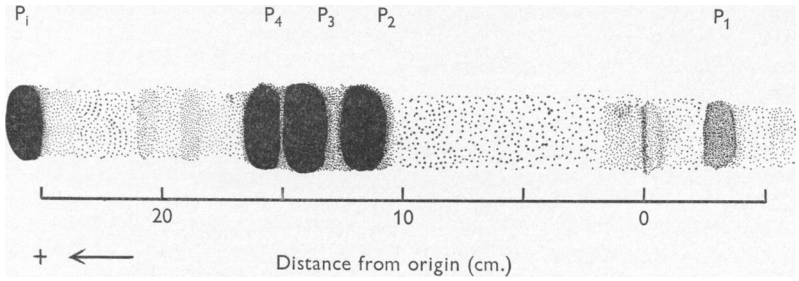

Fig. 1. Tracing of radioautograph obtained after ionophoresis (at pH 6·5 at 35 v/cm. for 1 hr. on Whatman 3 MM paper) of a peptic digest of ^{32}P-labelled phosphatase. The conditions of the digestion were as described in the text. P_i, Inorganic phosphate; P_1, P_2, P_3 and P_4, the main radioactive peptides.

sequence around it in order to economize in the amount of enzyme protein used. This is achieved by using a minimum of carrier phosphate. In preliminary experiments the highest specific radioactivity was obtained with 1 mc of [^{32}P]phosphate in the absence of carrier phosphate. The quantity of radioactive peptides thus obtained is limited by the amount of phosphate rather than by the amount of protein, and was too small for further handling since losses by adsorption to glass tubes, to pipettes and to paper were considerable. Since 1 mc of [^{32}P]phosphate was the maximum used for labelling, it was more convenient to use carrier phosphate to obtain larger quantities of peptides of lower specific radioactivity (1–2 μc/mg. of protein).

The ^{32}P-labelled phosphatase was subjected to proteolytic digestion with pepsin, after performic acid oxidation. Fig. 1 shows the ionogram of a 20 hr. digest. The distribution of radioactivity in each spot is shown in Table 1. The amount of inorganic phosphate (P_i) represents about 20 % of the total, and is probably due to contamination that could not be eliminated by washing the precipitated protein three times with acid acetone. The time-course of the proteolysis was also followed. After a short time (1½ hr.) the three main peptides, P_2, P_3 and P_4, are already present, but the P_2 spot is much stronger than P_4. After 16 hr. both spots are about equally strong. P_3 is the strongest at all times. The material remaining at the origin is initially considerable, but decreases with time. The peptides P_1, P_2, P_3 and P_4 represent nearly 70 % of the total radioactivity and more than 85 % of the radioactive peptide material. They were eluted, partially hydrolysed with acid and compared by ionophoresis at pH 3·5: Fig. 2 shows the distribution of radioactive peptides in the ionogram. It is possible to see that the main features of the four patterns are similar. It seems that the four peptic peptides are derived from the same site; but probably P_4 can arise additionally from P_2. However, the different intensities, or absence, of some

Table 1. *Peptides from the peptic digestion of ^{32}P-labelled phosphatase*

The percentage radioactivities were calculated by measurements of the radioactivity of the peptides of the ionogram shown in Fig. 1.

Bands	Radioactivity (% of total)
P_1	4·1
P_2	13·0
P_3	36·7
P_4	15·2
Phosphate (P_i)	19·4
Origin and minor spots	11·7

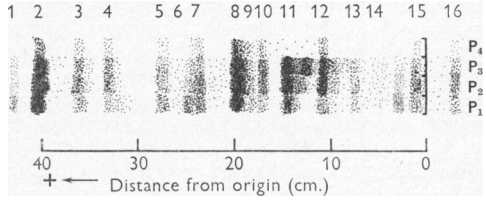

Fig. 2. Tracing of radioautograph obtained after ionophoresis (at pH 3·5 at 35 v/cm. for 2·5 hr. on Whatman no. 52 paper) of partial acid hydrolysates of peptic peptides (Fig. 1) derived from ^{32}P-labelled phosphatase. The numbers refer to the radioactive bands discussed below (see Fig. 3).

component bands indicate that the splitting by pepsin is near enough to the serine phosphate residue to yield peptides whose partial acid hydrolysates differ in their radioactive components.

Partial acid hydrolysis of ^{32}P-labelled phosphatase. The ^{32}P-labelled phosphatase was subjected to different conditions of partial acid hydrolysis. The distribution of radioactive peptides after ionophoresis at pH 3·5 is shown in Fig. 3. The proportion of radioactivity in each peptide band of the ionogram after 30 and 75 min. of hydrolysis with 5·7 N-hydrochloric acid is shown in Table 2. Band 2 was identified as serine phosphate by comparing it

with a serine phosphate marker during ionophoresis at pH 3·5. The ^{32}P-labelled phosphatase was hydrolysed with 5·7N-hydrochloric acid in a boiling-water bath, and samples were taken out at different times and subjected to ionophoresis at pH 3·5. The amount of ^{32}P in the main bands was determined by using an automatic recording chromatoscanner. The results are shown in Fig. 4 and the amount of each band is indicated in arbitrary units. The amount of material in band 2 increases continuously whereas all the others pass through a maximum. However, the maximum obtained for band 11 and 16 (and also 15 which is not included in the graph because quantitative estimation was hindered by contamination with material absorbed to the origin) is reached before 15 min., whereas bands 7 and 8 reach a maximum after 30 min. or more hydrolysis. The other bands are difficult to follow quantitatively because of the small amounts of material present at all times. Most of them reach a maximum before 30 min. and are not found in hydrolysates treated for longer times (see Table 2), except bands 1, 3 and 4, which reach a very low concentration and do not seem to change much even after 1·5 hr. of hydrolysis.

Most of the bands of the ionogram at pH 3·5 can be separated into other components by ionophoresis at pH 6·5. Fig. 5 (*a*) shows a radioautograph of a two-dimensional run at pH 3·5 and 6·5.

The two-dimensional peptide maps obtained were different when the order of the pH 3·5 and pH 6·5 runs was reversed. Some spots (those unnumbered in Fig. 5*a*) were absent when the second ionophoresis run was made at pH 3·5, and the intensity of some others was different, Table 3 shows their proportions as percentages of the total radioactivity recovered. It is evident that 8a, 11a, 12a and others are present in much lower yields if the first run is at pH 3·5 owing to the interconversion of some peptide (see below).

Structure of peptides. To determine the interrelations of the various bands shown in Fig. 5 (*a*), the material in them was subjected to a further partial acid hydrolysis (in 5·7N-hydrochloric acid at 100° for 45 min.) and the products were identified by ionophoresis, the original partial acid hydrolysate being used as a marker. Since most of the peptides were not pure, the comparison at a single pH was

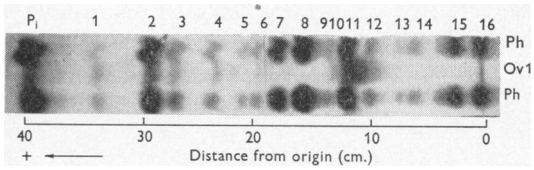

Fig. 3. Radioautograph obtained after ionophoresis (at pH 3·5 at 35 v/cm. for 2·5 hr. on Whatman 3MM paper) of partial acid hydrolysates of: Ph, ^{32}P-labelled phosphatase; Ov1, ^{32}P-labelled ovalbumin peptide 1. The partial acid hydrolysate of ^{32}P-labelled ovalbumin peptide 1 is known to contain P_i, serine phosphate (band 2) and the three forms of the dipeptide Asp-SerP (bands 1, 3 and 4) (Sanger, 1963).

Table 2. *Proportions of peptides from the partial acid hydrolysis of* ^{32}P*-labelled phosphatase*

The percentage radioactivities were calculated by measurements of the radioactivity of the peptides of ionograms run at pH 3·5. The mean values of three independent hydrolyses are given.

Bands	Conditions of partial acid hydrolysis: 5·7N-HCl for 30 min.	5·7N-HCl for 75 min.
	Radioactivity (% of total)	
Phosphate (P_i)	17·3	39·5
1	1·1	0·9
2	14·0	23·4
3	1·9	1·0
4	1·4	1·3
5	1·2	<0·5
6	1·3	<0·5
7	7·9	10·7
8	23·0	20·0
9 + 10	2·0	<0·5
11	13·0	1·0
12	2·0	<0·5
13	0·9	<0·5
14	1·5	<0·5
15	6·0	1·1
16	6·1	<0·5

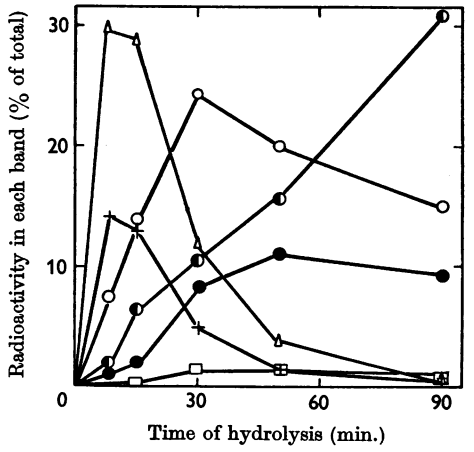

Fig. 4. Relative amounts of the main bands obtained after ionophoresis at pH 3·5 of the partial acid hydrolysis (in 5·7N-HCl at 100°) of ^{32}P-labelled phosphatase for different times. The numbering of the bands is as in Fig. 3: ◐, band 2; □, bands 3 and 4; ●, band 7; ○, band 8; △, band 11; +, band 16.

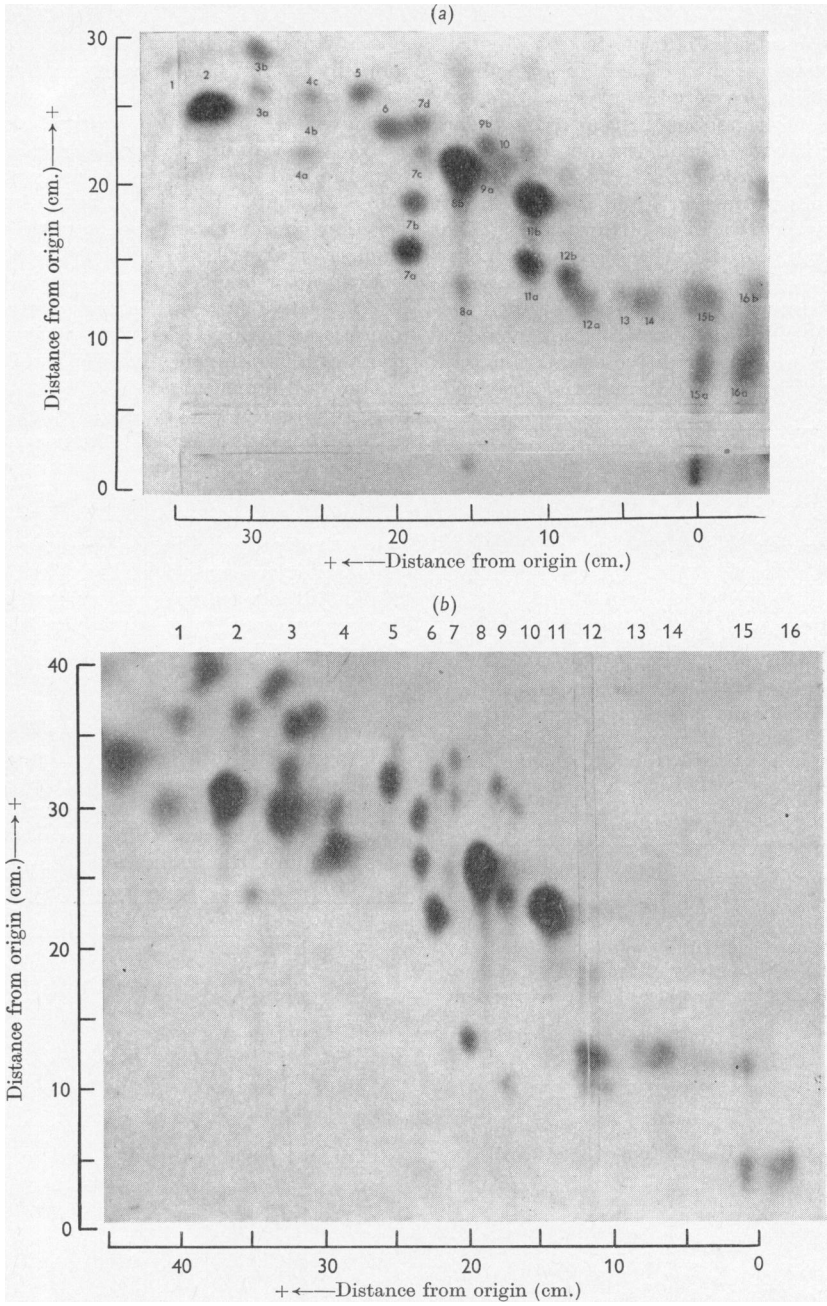

Fig. 5. (*a*) Radioautograph of two-dimensional ionograms (at 35 v/cm. on Whatman 3MM paper) of the partial acid hydrolysate of ^{32}P-labelled phosphatase. Hydrolysis was with 5·7 N-HCl at 100° for 30 min. P_i was allowed to run off the paper. The first dimension (abscissa) was at pH 3·5 for 2·5 hr., and the second dimension (ordinate) at pH 6·5 for 1·5 hr. (*b*) The partial acid hydrolysate was oxidized with periodate before the run. [A comparison of the periodate-treated partial acid hydrolysate with an untreated control was made by ionophoresis at pH 3·5, running both samples side by side. The only band that did not show up in the treated sample was 6. Bands 4, 7, 11, 12 and 16 were weaker in the treated sample. Some bands (e.g. 3) were stronger and other bands unchanged or not sufficiently strong to draw any conclusion.] The first dimension (ordinate) was at pH 6·5, and the second dimension (abscissa) at pH 3·5. Other conditions were as given for (*a*).

not sufficient to identify them. When this method of analysis was applied to phosphoglucomutase the impure bands at one pH could be resolved by the use of a single run at another pH. By comparing the results of the rehydrolysis in ionograms at two pH values it was possible to deduce the interrelations of the peptides (Milstein & Sanger, 1961). This was not possible with alkaline phosphatase.

The number of peptides was large and very few could be obtained pure by one-dimensional ionophoresis. Even after successive runs at pH 3·5 and 6·5 some peptides were not clearly separated from others. An additional complication was the presence of many peptides in very small amounts. To prepare the peptides two runs were necessary, and to study the products of their partial hydrolysis some of the component bands obtained at pH 3·5 were run again at pH 6·5. Adsorption by the paper was considerable and therefore the products present in low yields were likely to have been overlooked. This was particularly so when dealing with larger peptides from which several components were produced. Table 4 shows the products of the hydrolysis of the main peptides. The more acidic bands, 1, 3 and 4, gave only a few bands, indicating that they were very small peptides; 1 and 4a were interconverted during hydrolysis. The same was true of 7b and 8 although only a trace of 8 was obtained by hydrolysis of large amounts of 7b.

Table 3. *Proportions of peptides after two-dimensional ionophoresis of the partial acid hydrolysate of* ^{32}P*-labelled phosphatase*

Hydrolysis was with 5·7 N-HCl at 100° for 30 min. The values indicate the proportions (as percentages of recovered radioactivity) of each peptide spot (see Fig. 5*a*) after ionophoresis: A, at pH 3·5 in the first dimension, at pH 6·5 in the second dimension; and B, pH 6·5 in the first dimension, at pH 3·5 in the second dimension

Bands	Radioactivity (% of total) A	B
2	10·60	12·10
3a	0·37	0·66
3b	1·16	1·08
4a	0·41	0·65
4b	0·26	0·31
4c	0·40	0·68
5	1·09	1·33
6	1·49	1·77
7a	3·60	4·10
7b	1·62	1·85
7c	0·59	0·72
7d	0·91	0·83
8a	0·51	1·47
8b	25·00	25·50
9	0·93	0·88
10	0·57	0·79
11a	0·44	6·10
11b	17·60	16·40
12a	0·96	2·44
12b	2·13	2·50
13 + 14	1·86	1·44
15a	1·90	3·14
15b	2·55	0·98

Interconversion of serine phosphate dipeptides during acid hydrolysis has been previously described. The acidic Asp-SerP* dipeptide is present as three different spots in partial acid hydrolysates of chymotrypsin (Naughton *et al.* 1960). The dipeptide SerP-Gly is inverted under acid hydrolysis conditions (Naughton *et al.* 1960; Fölsh, Mellander & Strid, 1960).

Edman-degradation studies on band 8 indicated that serine phosphate was *N*-terminal, since large amounts of phosphate were produced (Table 5). Reasonable amounts of serine phosphate were produced from 7b, and although some phosphate was also liberated it seems that 7b is a dipeptide with *C*-terminal serine phosphate. Edman-degradation studies were also made with other bands. But in general only a small proportion of the peptides changed and some of the products ran in positions that did not match any of the known peptides, and the results were therefore difficult to interpret. When the partial acid hydrolysate was compared by ionophoresis at pH 3·5 with similar partial acid hydrolysates of proteins or peptides containing serine [^{32}P]phosphate in known sequences, some of the radioactive components were found to match perfectly (Figs. 3 and 6*a*). Thus bands 1, 3 and 4 were found to match with three derivatives of the dipeptide Asp-SerP, i.e. β-Asp-SerP, α-Asp-SerP and $\alpha\beta$-Asp-SerP respectively (see Naughton *et al.* 1960). Bands 7b and 8 on the other hand ran as the dipeptides Ala-SerP and SerP-Ala respectively (Fig. 6*a*). The radioactive bands corresponding to 7b and 8 (Fig. 6*a*) were cut out, sewn to another paper and run again at pH 6·5 (Fig. 6*b*). The result indicates that the main components of 7b and 8 are the dipeptides Ala-SerP and SerP-Ala respectively. Therefore it must be concluded that three dipeptides are present in the partial acid hydrolysate of ^{32}P-labelled phosphatase: Ala-SerP, SerP-Ala and Asp-SerP. Rehydrolysis of SerP-Ala produces Ala-SerP in high yields, but rehydrolysis of Ala-SerP produces only traces of SerP-Ala. Therefore Ala-SerP could be an artifact, but SerP-Ala should have been present in the original protein. There is also the possibility that Ala-SerP originates not only from the inversion of SerP-Ala but also from the protein itself. If this is so, two different sites of the attachment of P_i would be present. Figs. 1 and 2 show that bands 1, 3 and 4 (Asp-SerP derivatives) and band 8 (SerP-Ala) are present in the peptides obtained by peptic digestion which account for about 90% of the radioactive

* Abbreviation: SerP, serine phosphate.

Table 4. *Products of the rehydrolysis of radioactive bands*

Bands were isolated by ionophoresis at pH 3·5 and 6·5 and hydrolysed in 5·7N-HCl for 45 min. at 100°. They were again subjected to ionophoresis at pH 3·5, and when possible bands 4, 7 and 11 were sewn on to a new sheet of paper and rerun at pH 6·5; the products of the second run (a, b and c) are indicated with parentheses.

	Products of hydrolysis								
Band ...	1	2	3	4	5	6	7	8	11
Peptides									
1	+	+ +	.	(a) Trace	.	.	.	.	.
2	.	+ + +	.	.	.	.	.	.	.
3	Trace	+ + +	+ + +	.	.	.	(b) Trace	+	.
4a	+ +	+ +	.	(a) Trace	.	.	.	.	.
4b*	+	+ +	Trace	(b) Trace	.	.	?	?	.
4c	Trace	+	.	(c) Trace	.	.	(a) +	.	.
5	Trace	+ + +	+ +	(a) +	+	.	(b) + +	+ +	.
6	+	+ + +	+	+ +	Trace	Trace	(a) + +, (b) + +	+ + +	.
7a	+	+ + +	Trace	(a) +, (c) +	.	.	(a) + +	.	.
7b	.	+ + + +	.	.	.	.	(b) + + + +	Trace	.
7d	+	+ +	+	(a) +, (c) +	.	.	(a) +, (b) +, (d) Trace	+	Trace
8	.	+ + +	.	.	.	.	(b) + + +	+ + +	.
11a	.	+ + +	+	(a) Trace, (c) +	.	.	(a) +, (b) +	+ +	(a) +
11b	.	+ + +	.	.	.	.	(b) + + +	+ + +	(b) +

* Result of a single experiment. This band is the weakest detected in the hydrolysate and because of its very small yield was very difficult to study it further.

Table 5. *Products of the Edman degradation of radioactive bands*

Bands were isolated as indicated in Figs. 3 and 5; the bands were eluted and subjected to the Edman degradation followed by ionophoresis at pH 3·5. Identification of products was made by their position with respect to markers of the original partial hydrolysate.

Band	Products of hydrolysis
8	P_i (+ + +), peptide 8 (+ + +)
7b	P_i (+), peptide 2 (+), peptide 7b (+ + +)

peptides (Table 1). Therefore, if two different sites, about equally labelled, are present, they are near enough to one another not to be split into different peptides by pepsin.

Naughton *et al.* (1960) have shown that of the hydrolytic conditions they studied the minimum inversion of SerP-Gly took place at 37° in 5·7N-hydrochloric acid, and G. Fölsh (personal communication) has studied the conditions for the inversion of SerP-Leu, finding that inversion is minimal or absent when the partial hydrolysis is made at low temperature. When ^{32}P-labelled phosphatase was hydrolysed in 12N-hydrochloric acid at 37°, Ala-SerP was not detected in the hydrolysate (Fig. 7). Thus Ala-SerP is likely to be a by-product originating from the inversion of SerP-Ala present in the original sequence. This is also indicated by the fact that Ala-SerP is only present after hydrolysis of the peptides that contain SerP-Ala (Table 4).

The stability of the different forms of the Asp-SerP peptides has been discussed by Naughton *et al.* (1960), who have shown that at pH 6·5 the $\alpha\beta$-aspartyl form is converted into the β-aspartyl form. One would therefore expect such conversions when some bands isolated at pH 3·5 were run again at pH 6·5. This may be the reason why the two-dimensional ionophoresis map is cleaner when the pH 6·5 run is made before that at pH 3·5.

A further survey of this type of conversion was made. A partial acid hydrolysate of ^{32}P-labelled phosphatase was subjected to a two-dimensional ionophoresis, at pH 3·5 in both dimensions. Before the second run, the paper was soaked with buffer at pH 6·5 and left overnight in a humid chamber held at 30°. All spots should appear in a diagonal line, unless changes in the charge of the peptides have taken place during the incubation. That this has happened with several bands is shown in Fig. 8. Band 4 (presumably 4a) gives rise to material that runs as band 1, as would be expected from results obtained by Naughton *et al.* (1960). Band 9, after incubation, gives rise to a spot running as band 3, suggesting that 9a or 9b is the $\alpha\beta$-Asp-SerP-Ala tripeptide and that 3a is the β-aspartyl derivative (hydrolysis of band 3 gives both dipeptide derivatives; see Table 4). Band 5 may be the tripeptide α-Asp-SerP-Ala, since its mobility is slightly lower than that of a marker sample of the homologous α-Asp-SerP-Gly obtained from chymotrypsin, and on hydrolysis it gave both expected dipeptide derivatives (Table 4). After incubation at pH 6·5, band 11 produces a strong spot running in the position of band 6. Therefore 11a may be the $\alpha\beta$-aspartyl derivative of a tetrapeptide comprising the dipeptide 8 and the tripeptide 7a (see Fig. 9

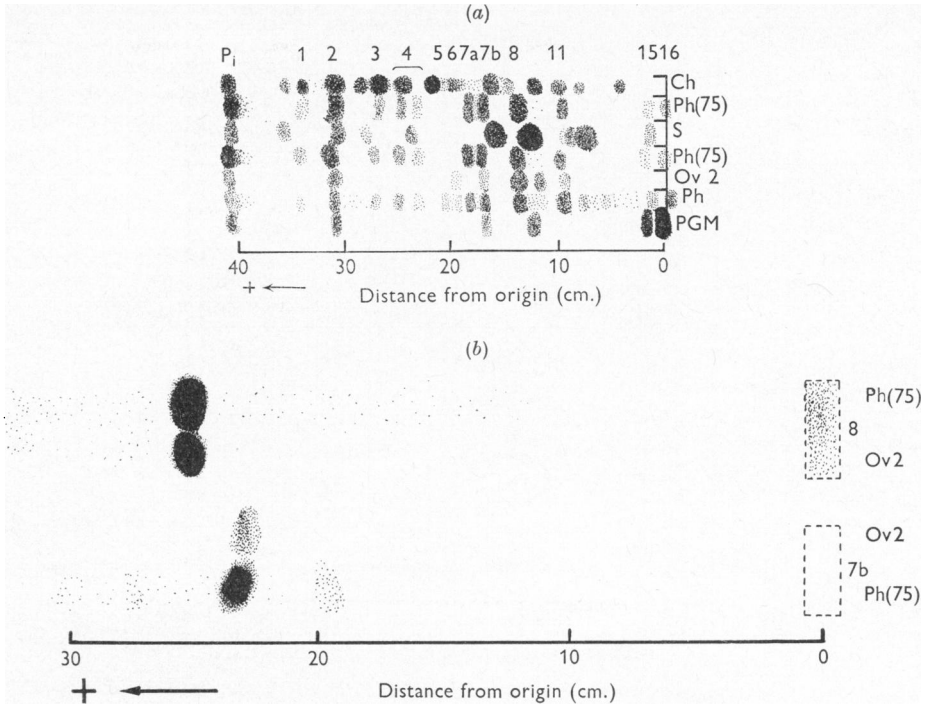

Fig. 6. (*a*) Tracing of radioautograph obtained after ionophoresis at pH 3·5 (at 35 v/cm. for 2·5 hr. on Whatman 3MM paper) of partial acid hydrolysates of: Ch, di-isopropoxy[^{32}P]phosphinyl-chymotrypsin (bands in positions 1, 3 and 4 are the β-, α- and $\alpha\beta$-aspartyl derivatives respectively of the dipeptide Asp-SerP; Naughton *et al.* 1960); Ph, ^{32}P-labelled phosphatase; Ph(75), ^{32}P-labelled phosphatase after partial acid hydrolysis for 75 min.; S, di-isopropoxy[^{32}P]phosphinyl-subtilisin (Sanger & Shaw, 1960); Ov2, ^{32}P-labelled ovalbumin peptide 2 (bands in position 7b and 8 are Ala-SerP and SerP-Ala respectively; F. Sanger, unpublished work); PGM, ^{32}P-labelled phosphoglucomutase (band in position of 7b is Ala-SerP; Milstein & Sanger, 1961). [The pattern of the ionograms at pH 3·5 was extremely sensitive to minor changes of pH. When the pH is lower, band 4a run ahead of 4b, and 4c and 7a ahead of 7b. At higher pH values the reverse was true. Different patterns from the one at pH 3·5 were obtained on ionophoresis at pH 3·1 and at pH 4·0 (see Fig. 7).] (*b*) Tracing of radioautograph obtained after ionophoresis on a second dimension (at pH 6·5 at 35 v/cm. for 2 hr.) of band 7b and 8 from (*a*) common to Ph(75) and Ov2. The bands were sewn on Whatman 3MM paper as indicated by the broken lines.

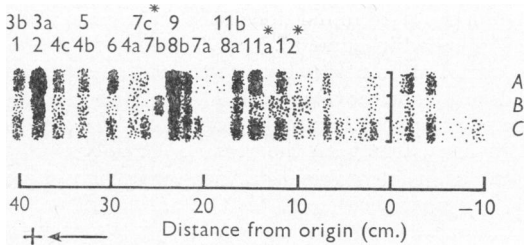

Fig. 7. Tracing of radioautograph obtained after ionophoresis at pH 4·0 (at 20 v/cm. for 6·5 hr.) of partial acid hydrolysates of ^{32}P-labelled phosphatase: *A*, in 12 N-HCl at 37° for 5 days; *B*, in 5·7 N-HCl at 100° for 30 min.; *C*, in 12 N-HCl at 37° for 2 days. The positions of the bands were located from an ionogram at pH 4·0 of the isolated bands obtained after ionophoresis at pH 3·5. The exact positions of bands marked * are somewhat uncertain.

and Table 4). Band 6, the product obtained after incubation of 11, may then be the β-aspartyl derivative. Other spots departing from the diagonal are also present (Fig. 8), some of which may be produced by decomposition. The presence of unexpected radioactive derivatives has been observed in such studies, and P_i is produced on standing. Others could originate from the $\alpha\beta$-aspartyl derivatives of bigger peptides, most of which are decomposed during the treatment. When bands 4a, 11a, 12a and 12b were isolated at pH 6·5, incubated for 20 min. with 5·7 N-hydrochloric acid at room temperature and, after elimination of the acid, identified by ionophoresis at pH 3·5, it was found that 4a was completely transformed into 1, 11a almost completely into 6, 12a completely into 9, and 12b completely into 7. Most of the material in bands 15

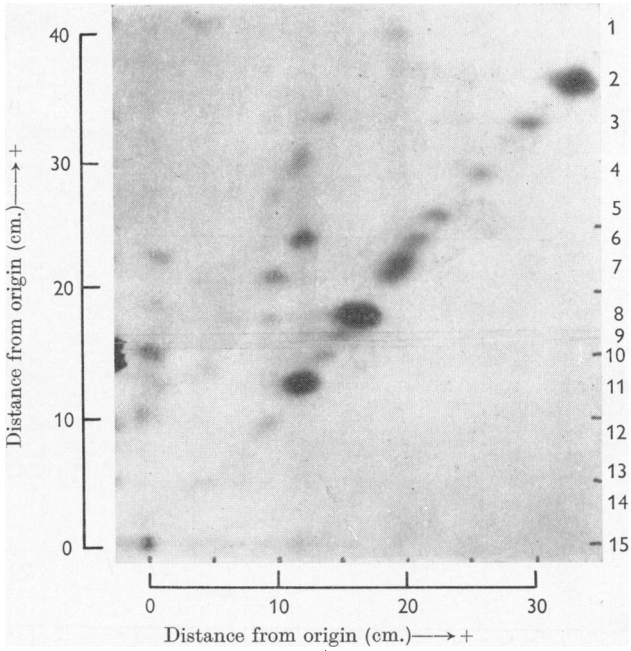

Fig. 8. Radioautography obtained after two-dimensional ionophoresis, both dimensions being run at pH 3·5, of a partial acid hydrolysate of ^{32}P-labelled phosphatase. After the first run (at 35 v/cm. for 2·2 hr. on Whatman 3MM paper) the dried strip of paper was sewn to another sheet of the same paper, carefully moistened with pyridine–acetate buffer, pH 6·5, and left for 18 hr. in a closed tray saturated with the same buffer. The paper was then allowed to dry and the second dimension was then run (at 35 v/cm. for 2·5 hr.).

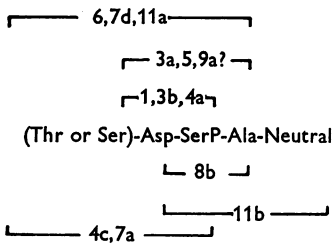

Fig. 9. Tentative scheme of the structure of the main peptides present in the partial acid hydrolysate of ^{32}P-labelled phosphatase.

and 16 gave rise to other bands, suggesting the presence of bigger peptides containing the ring form of aspartic acid. These may include some amino acids in addition to those present in the peptides described above.

A diagram showing the suggested sequence of the main peptides is shown in Fig. 9. The minor bands should be considered as tentative. The rehydrolysis of the bands not included in Table 4 gave unreliable results, probably because some bands (e.g. 12a and 12b) are not well separated and the yields are low. The products of the hydrolysis were present as weak spots, and the possibility that peptides may not be recognized increases with the size of the peptide. This should be considered as the main source of error. Nevertheless, 15a and 15b did not give 8 on hydrolysis, there was a clear spot in the position of band 7 (which was not further identified), and bands in positions 1, 3 and 4 were also present. It may well be that an extra amino acid on the *N*-terminal side of the diagram in Fig. 9 was included in band 15.

Several attempts to identify the unknown amino acids present in the tripeptides 11b and 7a were made. The electrophoretic mobility of 11b at pH 6·5 and 3·5 is consistent with the presence of only neutral amino acids in combination with serine phosphate. The chromatographic R_F of the unknown neutral amino acid (Milstein & Sanger, 1961) was measured. This value is not high in butanol–acetic acid–water–pyridine, suggesting that it is not leucine, isoleucine or phenylalanine. However, the errors were too high to throw more light on the nature of the neutral amino acid at the *C*-terminus of the sequence shown in Fig. 9.

Periodate oxidation of the partial acid hydrolysate showed definite changes with some bands

(Fig. 5*b*). After periodate oxidation bands 4c, 7a, 11a, 6, 9b and 10 completely disappeared. All these bands should contain threonine or serine in the *N*-terminal position (Sanger & Shaw, 1960; Milstein & Sanger, 1961). That is the reason why in Fig. 9 threonine or serine has been placed before aspartic acid. Other bands are not so definite. It seems that 7c and 7d are no longer present.

Characterization of the hydrolysate under different ionophoretic conditions. Ionophoresis at pH 3·5 gave mostly contaminated peptides which were purified at pH 6·5. Ionophoreses at pH 2·5, at pH 3·1 and at pH 6·5 were tried, but they were no better than that at pH 3·5 and the peptides were still contaminated. The use of pH 4·0 (allowing P_i to move off the paper) might be more convenient for comparative purposes. In Fig. 7 ionophoresis at pH 4·0 of the partial hydrolysates made with 5·7N-hydrochloric acid for 30 min. at 100° and with 12N-hydrochloric acid at 37° for 2 and 5 days are compared. The advantage of the pH is shown by the fact that there are differences not detected at pH 3·5. The two tripeptides (bands 7a and 11b) in which all the amino acids of the central pentapeptide are included are present at pH 4·0 as uncontaminated spots. This was only true with very long runs (about 6–8 hr. at 20v/cm.).

Other conditions of partial acid hydrolysis. Specific cleavage of bonds involving aspartate has been observed by some authors (see Witkop, 1961) with dilute acids. Some experiments were made to obtain peptides, free of the complexity of the aspartyl derivatives, that might include some extra amino acids on the *C*-terminal side of the serine phosphate residue. Fig. 10 shows the hydrolysates made with 0·033N-hydrochloric acid at 105° for various times. Phosphate is formed in rather high proportions, which is very inconvenient because the yield of peptides is very low. The O–P bond seems to be weaker than the peptide bond under these conditions, and negligible amounts of serine phosphate are formed. The strongest peptide bands present after the protein-like material has been hydrolysed are 11, 8 and 7. The material in band 11 was run again at pH 6·5 and found to contain two components. The main one was 11b and the other was a faster-moving band. Band 7 was identified as the same as 7b obtained at pH 6·5. Most of the peptides containing the Asp-SerP bond are, therefore, absent. The only exception seems to be band 3, but this was not investigated further. The method seems to be good for preferential splitting of aspartyl bonds, but did not show complete specificity since a fair amount of the dipeptide SerP-Ala is present. The bond SerP-Ala–X was split, X being a neutral amino acid. Somewhat similar considerations may be applied to hydrolysis with 10% acetic acid. By using shorter times of treatment with acetic acid, however, very slow-moving bands are present in better yields.

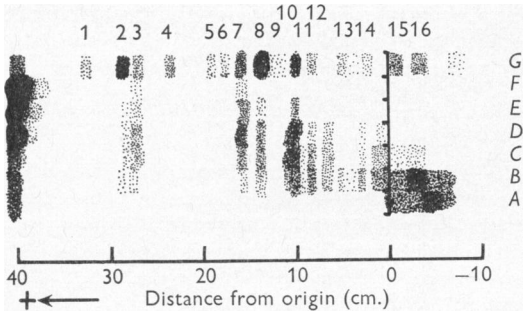

Fig. 10. Tracing of a radioautograph obtained after ionophoresis (at pH 3·5 at 35v/cm. for 2·5 hr.) of partial acid hydrolysates of ^{32}P-labelled phosphatase under different conditions: 0·033N-HCl at 105° for *A*, 0 hr.; *B*, 2 hr.; *C*, 5 hr.; *D*, 10 hr.; *E*, 24 hr.; *F*, 42 hr.; *G*, 5·7N-HCl at 100° for 30 min.

DISCUSSION

The work described was an attempt to identify the sequence around the serine [^{32}P]phosphate residue in alkaline phosphatase by using methods similar to those used previously (Naughton *et al.* 1960; Milstein & Sanger, 1961). The amount of protein needed for this study was again very small, and from this point of view phosphatase is an ideal enzyme, because it can be labelled to any required specific radioactivity. However, owing to adsorption losses, there is a lower limit to the amount of protein that can be handled. A specific radioactivity of 1–2 μc/mg. of protein was used, and it proved high enough, since considerable work could be done with less than 1 mg. of enzyme.

The stability of the various peptide bonds towards acid hydrolysis is very different, and a very low yield of some peptides has been observed. Yields of the dipeptides of the general structure Asp-SerP were considerably lower than those of the same peptides obtained under the same conditions from chymotrypsin (Naughton *et al.* 1960). As a consequence of these low yields, the study of the sequence was confusing and that of the interrelationship of the peptides handicapped.

This and artifacts, such as the inversion of SerP-Ala, show how unreliable analysis of a partial acid hydrolysate may be. Some experiments were made with proteolytic enzymes to produce small peptides. Pepsin (see Fig. 2) seems to produce a peptide (P_4) from which the component 11b is not obtained after partial acid hydrolysis, suggesting that it ends in SerP-Ala (see Fig. 9). Carboxypeptidase (C. Milstein, unpublished work) did not

seem to produce appreciable splitting near the serine phosphate residue. This seems to be due to the presence of the strongly charged phosphate residue (Schwartz, Crestfield & Lipmann, 1963).

Naughton *et al.* (1960) have studied the partial hydrolysate of di-isopropoxy[^{32}P]phosphinyl-chymotrypsin, and found about 20 peptides derived from the sequence Asp-SerP-Gly. They suggested that some of these arose from a conversion of the α-aspartyl sequence into the αβ- and β-aspartyl forms. By using a similar explanation it is possible to interpret the main peptides derived from the partial acid hydrolysate of ^{32}P-labelled alkaline phosphatase. The tentative structure of the peptides present in the hydrolysate (Fig. 9) has been mostly based on the rehydrolysis technique and on the electrophoretic mobility of the peptides. In Fig. 9 two tripeptides are shown with the sequence (Thr or Ser)-Asp-SerP; one is 7a, which on rehydrolysis gives the Asp-SerP pattern; it has an electrophoretic mobility curve that shows an acidic group to be lacking (C. Milstein, unpublished work), suggesting it is the αβ-aspartyl derivative. Its yield and stability are however much higher than those found for any other αβ-aspartyl derivative. There should be a third peptide containing the same amino acids, but it could not be located. One possibility could be that only one aspartyl derivative of such a peptide can reversibly form the ring derivative with the greater stability.

The radioactive peptides in the partial acid hydrolysate of alkaline phosphatase seem to account for at least a pentapeptide sequence that contains serine phosphate with serine or threonine as the *N*-terminus and a neutral amino acid as its *C*-terminus. Schwartz *et al.* (1963) have proposed the sequence Thr-Asp-Ser-Ala-Ala around the reactive serine residue in alkaline phosphatase from *E. coli*, in agreement with the results reported above. Engström (1962*b*), on the other hand, has published a comparison of the hydrolysates of ^{32}P-labelled phosphatases from *E. coli* and from calf intestinal mucosa. It is possible to compare his results with those in the present paper since the conditions were fairly similar. The peptides of the sequence Asp-SerP-Ala are common to both. Also band 11 seems to be present in both preparations, suggesting that the similarity extends to at least a tetrapeptide sequence. However, band 6 and one of band 4 seems to be absent in the hydrolysate of the intestinal enzyme, and also some of the slowest-moving bands are different. This indicates differences near the serine residue. It could be that the *N*-terminal end of the pentapeptide from the intestinal enzyme is different.

Alkaline phosphatase is another enzyme with an active serine residue contained in a sequence of the type (Asp or Glu)-Ser-(Gly or Ala). The similarity, although striking, is not, however, as general as it was sometimes believed (for a general table of sequences around reactive serines see Sanger, 1963). Different sequences have also been found in enzymes with similar enzymic activities. These 'exceptions' indicate that the active serine residue is not necessarily included in such an amino acid sequence. As pointed out by Naughton *et al.* (1960), the similarity between the proteases may be due to the biological origin of the proteins. These authors suggested that 'some primitive organisms contained a single protease and that, during the evolutionary process, three enzymes with different specificities (trypsin, chymotrypsin and elastase) have developed from it'. This, however, does not explain the similarity with the enzymes thrombin, liver aliesterase and pseudocholinesterase. The fact that alkaline phosphatase contains the 'intermediate' sequence between the Asp-Ser-Gly and the Glu-Ser-Ala sequences makes the similarity more significant. It is possible that in very primitive organisms proteins had a wide range of enzymic activity. When specificity developed, those sequences of amino acids essential for enzymic activity were kept intact, unless a related amino acid could replace one of the original members. The finding in alkaline phosphatase of a sequence containing aspartate as in trypsin, chymotrypsin, elastase and thrombin, and alanine as in liver aliesterase and pseudocholinesterase, emphasizes the possibility of a common origin. If this is so, it would not be surprising to find a reactive serine residue in a sequence Glu-Ser-Gly. The completely different sequences found in subtilisin and phosphoglucomutase could be considered to have evolved entirely independently, and may in turn have given rise to families of enzymes containing common sequences. One should then hope to find reactive serine residues in sequences similar to those found in phosphoglucomutase or subtilisin. Very recently mould protease has been found to contain a sequence similar to that of the latter enzyme (Sanger, 1963).

SUMMARY

1. Alkaline phosphatase from *Escherichia coli* was labelled with ^{32}P of high specific radioactivity by the incorporation of inorganic [^{32}P]phosphate, and radioactive techniques were used to study the sequence around the site of incorporation of phosphate.

2. The ^{32}P-labelled phosphatase was subjected to partial hydrolysis with pepsin and with acid. The ^{32}P-labelled peptides were purified by paper ionophoresis. The peptides were not resolved in one dimension, and a very complex pattern was obtained in two dimensions. Their interrelationships

were studied by subjecting them to a further partial hydrolysis.

3. A dipeptide Ala-SerP was identified and shown to be derived from the inversion of SerP-Ala present in the original protein (SerP refers to serine phosphate).

4. The presence of Asp-SerP dipeptides in three forms was detected. A diagonal ionophoretic technique was used to study the interconversion of the aspartate-containing peptides.

5. On the bases of ionophoretic mobilities and periodate oxidation of the radioactive peptides it was concluded that peptides accounting for the sequence (Thr or Ser)-Asp-SerP-Ala-neutral amino acid were present in the partial acid hydrolysate.

6. The hydrolysis with dilute acid gave a preferential splitting at the aspartate residue, but phosphate was also produced in high yields. Splitting at sites other than aspartate residues was also detected.

7. The results are discussed in connexion with the use of tracer techniques in determinations of amino acid sequences. The similarity between the sequence described and those of other hydrolytic and proteolytic enzymes is also discussed.

I am grateful to Mrs M. F. de Strasnoy for her assistance at different stages of this work, and to the Consejo Nacional de Investigaciones Cientificas y Tecnicas (Republica Argentina) for financial support during part of this work.

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APPENDIX

The Amino Acid Sequence Around the Reactive Serine Residue in Alkaline Phosphatase of *Serratia marcescens*

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(*Received* 3 *December* 1963)

A comparative study of the amino acid sequence around the reactive serine residue in alkaline phosphatases of *Serratia marcescens* and *Escherichia coli* was made by using the technique of matching radioactive peptides. Alkaline phosphatase from *Serratia* has been studied by Signer, Torriani & Levinthal (1961) and found to be similar in many respects to the *E. coli* enzyme. Significant differences were, however, detected, mainly in the isoelectric point and the 'fingerprints' of both proteins.

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The enzyme from *Serratia* was purified by the same procedure as used for the enzyme of *E. coli* by Milstein (1964). Two preparations containing about 0·5 mg. of enzyme, one of *E. coli* and one of *Serratia*, were labelled at the same time by the general procedure described by Milstein (1964), except that 1 mg. of carrier γ-globulin was added after the incubation with [^{32}P]phosphate. The precipitated protein in acid acetone was suspended in distilled water and dialysed overnight against distilled water. The insoluble γ-globulin was centrifuged down and the soluble radioactive protein was used, after drying over sodium hydroxide. The radioactive residues were partially acid hydrolysed with constant-boiling hydrochloric acid for 30 min. in a boiling-water bath. After complete elimination