† Established Investigator of the American Heart Association.

‡ This may be seen from the values in parentheses in Table 1, part 1.

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*PHOSPHATE INCORPORATION INTO ALKALINE PHOSPHATASE OF E. COLI**

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Serine phosphate has interested us for a long time.[1, 2] More recently, the function of serine as a component of an active center in enzymes has received much attention.[3, 4] The quality of the hydroxyl group in serine is obviously variously modified by neighboring amino acids and, more generally, by structural features of the molecular environment.[5, 6] Now, the reports of Ågren,[7] and of Engström[8] on the isolation of serine phosphate from phosphate-incubated mammalian alkaline phosphatase make it seem likely that serine is the active center of this enzyme. The availability of the phosphatase of *E. coli* in a rather pure form[9] made us hopeful that we might here have an opportunity to obtain precise information on the situation of the reactive serine in this enzyme. This seemed particularly desirable since the genetic characterization of the phosphatase is far advanced.[10–12] We hoped eventually to map the amino acid sequence at the active center by marking serine in the enzyme with radioactive phosphate. In accord with observations on other phosphatases, we found that after incubation with inorganic phosphate, serine phosphate can be isolated from *E. coli* phosphatase. Similar results with this enzyme were obtained independently by Engström.[13]

Preparation of the Enzyme.—The enzyme was purified by a modification of the method outlined.[9] Because of these changes, we present the procedure in some detail. A constitutive strain of *E. coli* K 12 (C_4P^+) was grown and harvested as described.[14] The cell paste, weighing 2.4 kg, was homogenized in 400 ml of water in a Waring blendor at 4°. About 1 liter of acetone at −20° was added to the smooth paste, and briefly blended. The resulting suspension was rapidly poured into 16 liters of acetone at −20°, and then filtered. The filter cake was washed with multiple small amounts of cold acetone totaling 4 liters, and then with 5 lb of ether at −20°. When the temperature of the filter cake rose to 4°, the material was spread out thinly on blotting paper to dry at room temperature.

The dried powder was extracted with 2 liters of 0.1 *M* Tris HCl buffer at pH 7.4 with 0.01 *M* $MgSO_4$ and 1 mg DNase at room temperature for 2 hr. After centrifugation, the extract was fractionated with ammonium sulfate and the precipitate from the 30–60 per cent saturated solution was retained. Ammonium sulfate was removed by dialysis against 0.1 *M* Tris HCl buffer and 0.01 *M* $MgSO_4$. The resulting solution was heated for 15 min at 75° and the precipitate discarded. The supernatant was incubated with RNase (10 μg/ml) at 30° for 2.5 hr, and exhaustively dialyzed first against 0.1 *M* Tris HCl buffer pH 7.4 and 0.01 *M* $MgSO_4$ and then against a ten-fold dilution of the buffer. The dialyzed solution contained 10.4 mg of protein per ml as determined by the Lowry method,[15] using albumin as a standard, and 9.6 mg of alkaline phosphatase per ml as estimated from the turnover number for *p*-nitrophenyl phosphate reported[9] using 80,000 as the molecular weight. Some of this material was chromatographed on DEAE-cellulose as described.[9]

Partial Identification of Hydrolysis Products of Phosphate-Treated Enzyme.—The enzyme was hydrolyzed for 18 hr in 2 *N* HCl in a boiling water bath[1, 7] and the products of hydrolysis were electrophorized. In Figure 1, an electropherogram is compared with its corresponding radioautograph. The picture shows the presence of serine phosphate and of three more slowly moving radioactive compounds, presumably serine phosphate containing peptides 1, 2, and 3. The radioactive material overlapping authentic serine phosphate was again electrophorized at pH 1.9, 4.7, 6.5, and 8; its mobility always corresponded exactly to that of authentic serine phosphate. The other radioactive compounds that appear on Figure 1 were eluted and further hydrolyzed for 6.5 hr. Radioautographs of the electropherogram of the products of such hydrolyses are shown in Figure 2. Peptide 3, which was obtained in smallest yield, is hydrolyzed to serine phosphate and peptides 1 and 2 (Fig. 2, Series 1). Peptide 1 yields serine phosphate and peptide 2, as can be seen in Figure 2, Series 2.

These experiments show the three peptides to be partly interconvertible: peptide 3 yields 1 and 2; 1 yields 2; and all of them partially hydrolyze to serine phosphate. Further preliminary information on the localization of serine phosphate was obtained by brief hydrolysis of the enzyme protein in 6 *N* HCl at 100°. After 10, 20, and 30 min of hydrolysis, approximately a dozen distinct radioactive compounds appeared on electrophoresis in addition to serine phosphate (Fig. 3).

In view of the presence of 33 serines in this phosphatase,[16] it was important to determine whether phosphate is fixed to a special serine. Trypsin digestion yields 35 distinct peptides,[11, 17] as we have confirmed. A tryptic digest of phosphorylated

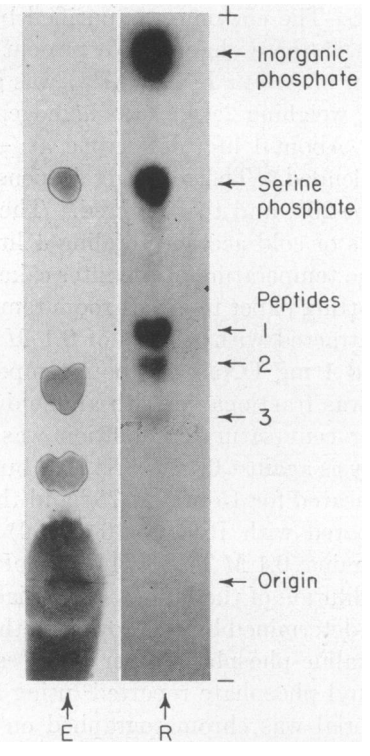

FIG. 1.—Electropherogram and corresponding radioautograph of the products from phosphorylated alkaline phosphatase after partial acid hydrolysis in 2 *N* HCl. Incubation: 150 mg of enzyme with 0.1 μM phosphate, specific activity 5 mC/μM, in 0.1 *M* Tris HCl buffer pH 8, and 0.001 *M* $MgSO_4$ for 5 min at 34°. Protein was precipitated by the addition of 0.2 ml 50% TCA and washed twice with iced 5% TCA and once with ether. The protein was hydrolyzed in 5 ml 2 *N* HCl on a steam bath, with refluxing, for 18 hr. The hydrolysate was lyophilized to dryness and then dissolved in 0.5 ml of 0.1 *N* acetic acid. Authentic serine phosphate was added as an internal standard, and 20 μl were applied to wet Whatman 3 MM paper for electrophoresis at pH 3.3 (acetic acid:pyridine:water/100:5:895) for 2.5 hr at 3 kv on a water-cooled copper plate. *E* is the electropherogram stained with ninhydrin. *R* is the corresponding radioautograph made with clinical X-ray film stapled to the paper and exposed for 30 min. All electropherograms are oriented so that the positive electrode is toward the top of the page.

phosphatase yielded predominantly one radioactive peptide, as shown in Figure 4, indicating that the reactive serine is located in a single region of the enzyme molecule. In addition to the main component, some undigested radioactive material remained at the origin, and a small fraction of the radioactive material moved more rapidly to the negative electrode than did the major radioactive tryptic product. This last fraction seems to consist of peptides which also appear on digestion of the phosphatase by chymotrypsin. It is probable that the faster-moving compound is not a product of trypsin digestion but is due to contamination of the trypsin with chymotrypsin, since commercial trypsin preparations are known to be so contaminated. The major tryptic product remains a single band after two-dimensional electrophoresis in two different systems, or after two-dimensional electrophoresis followed by paper chromatography.[18] When the major tryptic product was hydrolyzed for 18 hr in 2 *N* HCl at 100°, an electropherogram of the hydrolysate showed serine phosphate and the same three peptides seen in the hydrolysate of phosphate-treated whole phosphatase, as shown in Figure 1.

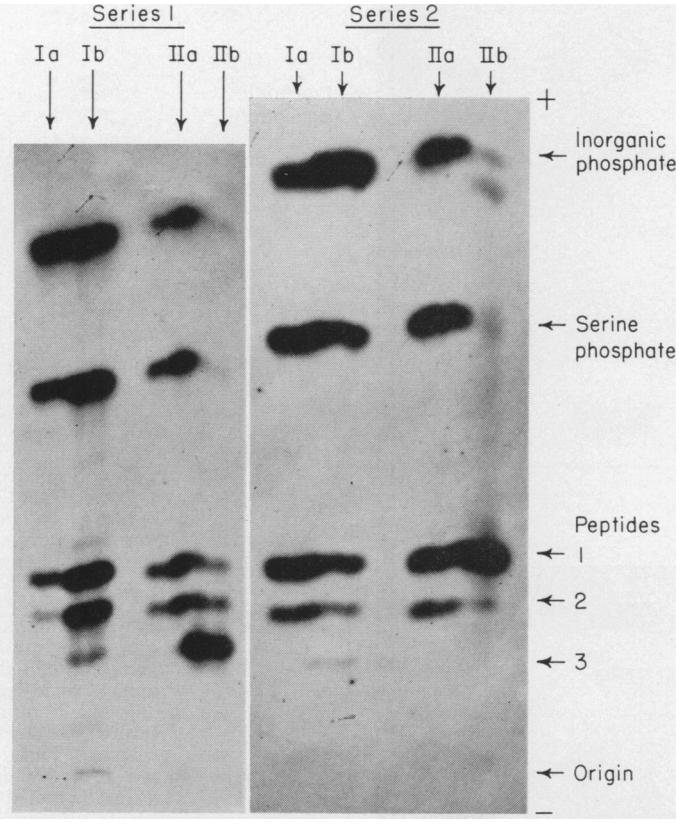

FIG. 2.—Comparison of electropherograms of radioactive peptides from acid hydrolysates of phospho-enzyme and products of their further hydrolysis. The radioactive material from areas 1 and 3 in Fig. 1 was eluted with water and aliquots were further hydrolyzed in 2 *N* HCl on a steam bath for 6.5 hr. *Series 1.* (I*a*) 18-hr hydrolysate; (I*b*) products of further hydrolysis of isolated peptide 3; (II*a*) same as I*b*; (II*b*) peptide 3 as isolated from 18-hr hydrolysate. *Series 2.* (I*a*) products from the further hydrolysis of peptide 1; (I*b*) original 18-hr hydrolysate (as in Fig. 1); (II*a*) same as I*a*; (II*b*) peptide 1 eluted from chromatogram of 18-hr hydrolysate. The amount of radioactivity in the 18-hr hydrolysate applied to the paper was adjusted to a level matching the amount in each peptide sample. The conditions of electrophoresis were the same as those described for Figure 1. Exposure of the X-ray film was 3 days for the petptide 1 series, and 4 weeks for the peptide 3 series.

Some Chemical Characteristics of Phosphate Binding.—Alkaline phosphatase is extraordinarily resistant to denaturing reagents. After precipitation with cold 5 per cent TCA,† approximately 50 per cent of the enzyme activity can be found in the washed precipitate. Enzyme activity can be recovered even after TCA precipitation, followed by dissolving in 88 per cent formic acid for 30 min at 0° and precipitating again with 5 per cent TCA. Reflecting this resistance to denaturation of the enzyme protein, incubation of an enzyme suspension in 5 per cent TCA with radioactive phosphate resulted in definite incorporation into the serine. This unusual stability has made it difficult to collect exact and numerically reliable data. No procedure in our hands denatures instantly, arresting phosphate incorporation and its reversal. The only procedures found to result in irreversible denaturation of the enzyme with respect to its hydrolytic activity are autoclaving, boiling with acid, and reduction with thioglycolate followed by alkyl-

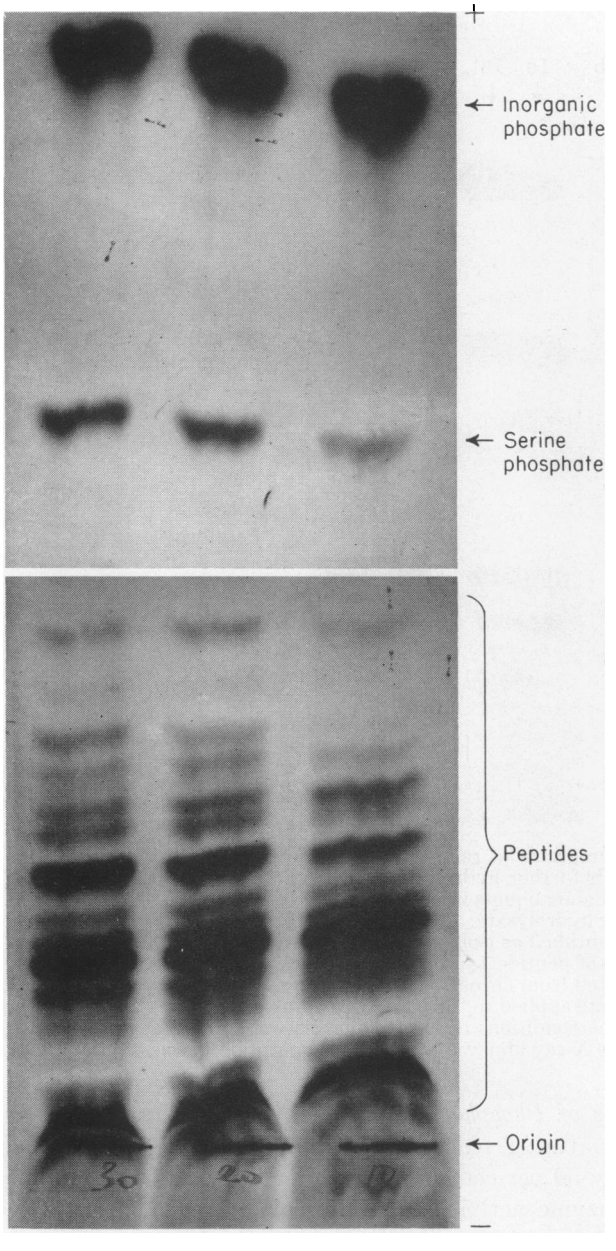

FIG. 3.—Radioautograph of an electropherogram of the acid hydrolysis products of P^{32}-phosphorylated alkaline phosphatase. 24 mg of enzyme were incubated for 5 min at 30° in 0.1 *M* Tris HCl buffer, pH 8 with 0.08 *M* phosphate specific activity 12.5 $\mu C/\mu M$. After incubation, the protein was precipitated with acetone at −20°, and the precipitate washed twice with cold 5% TCA and twice with ether-acetone (3:1). The residue was then autoclaved for 15 min. The autoclaved material was suspended in 6 *N* HCl and placed on a steam bath. At 10, 20, and 30 min, 0.5-ml samples were removed and dried in a dessicator. These were redissolved in a small amount of water and applied to the paper for electrophoresis at pH 3.3 for 5.5 hr at 4.2 kv. X-ray film was exposed for 16 hr to obtain the radioautograph.

ation with iodoacetate.[17] Enzyme denatured by autoclaving or by the reduction-alkylation procedure lost the capacity to incorporate phosphate.

The data reproduced in the following tables are presented in spite of the variability already mentioned so that some preliminary notion might be given concerning the chemical characteristics of phosphate fixation. Incorporation of phosphate is favored by acid conditions. The incubation at pH 5.4 in some of our experiments, using only a ten-fold excess of phosphate to enzyme, results in nearly 1 mole of phosphate fixed for each mole of enzyme (Table 1), while under similar

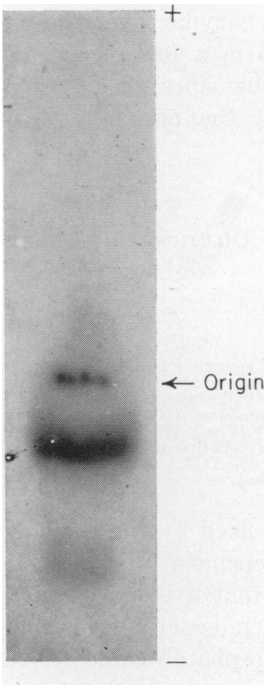

FIG. 4.—Radioautograph of an electropherogram of a tryptic digest of P^{32}-phosphorylated alkaline phosphatase. Phosphorylated enzyme was prepared as described for Figure 3. After autoclaving, the protein was reduced with thioglycolate and then alkylated with iodoacetate[17] in 8 *M* urea. The reduced material was dialyzed against 0.01 *M* NH_4HCO_3, and after drying in a dessicator, was suspended in 2 ml of 0.05 *M* NH_4HCO_3 containing 0.2 mg of crystalline trypsin (Worthington). After digestion for 1 hr at 37°, 1 *M* acetic acid was added and the digest dried in a dessicator. It was then resuspended in 0.2 ml of water, 10 μl of which were applied to the paper for electrophoresis at pH 4.6 at 3 kv for 3.5 hr. The X-ray film was exposed for 16 hr, and the resulting radioautograph represents all of the radioactivity on the paper, with the exception of a small amount of inorganic phosphate.

TABLE 1

KINETICS OF PHOSPHATE INCORPORATION AT pH 8 AND pH 5.4

	cpm/0.175 μM enzyme ($\times 10^{-4}$)	Moles phosphate bound/moles enzyme
pH 8 (0°)		
10 seconds	3.5	0.045
1 minute	3.6	0.047
2 minutes	6.2	0.08
5 minutes	3.8	0.05
10 minutes	8.0	0.10
pH 5.4 (30°)		
15 seconds	66	0.86
1 minute	62	0.8
2 minutes	62	0.8
7 minutes	77	1.0

The tests were made in 0.2 *M* Tris acetate buffer, 0.001 *M* $MgSO_4$, with 1 μM/ml of phosphate having a specific activity of 4.4×10^6 cpm/μM, in a total volume of 8 ml. At timed intervals, 2-ml samples containing 0.175 μM enzyme were taken. Protein was precipitated by the addition of 0.2 ml of 50% TCA and the precipitate washed twice with 5% TCA. The precipitate was dissolved in 1 ml of 88% formic acid, then diluted with iced water to 9 ml and precipitated once more by the addition of 1 ml of 50% TCA. This precipitate was transferred to planchets for counting in 88% formic acid. For checks on this isolation procedure, see Table 3.

conditions at pH 8, only 0.1 mole per mole of enzyme or less was fixed. The reaction between phosphate and enzyme is very fast, even at 0°, since probably complete equilibration is reached in a few seconds (Table 1). Phosphorylation of enzyme appears to be reversible since, on dilution, phosphate is released. After 37-fold dilution, only $^1/_{15}$ of the originally incorporated phosphate remained (Table 2).

TABLE 2

EFFECT OF DILUTION ON PHOSPHATE FIXATION

Incubation	cpm/0.15 mg enzyme	Moles phosphate/ mole enzyme
First incubation	76,000	0.1
After dilution	5,000	0.0065

In a first period, 0.3 μM of enzyme was incubated for 5 min at 30° in 0.2 *M* Tris acetate buffer pH 8, and 1 μM/ml of phosphate with a specific activity of 5.25×10^6 cpm/μM, in a total volume of 2 ml. At the end of this period, 1 ml was pipetted into 0.1 ml of 50% TCA and kept on ice. Immediately, 36 ml of 0.1 *M* Tris buffer pH 8, warmed to 30°, were added to the remaining 1 ml. After 10 min of further incubation, the tube was cooled in ice and 4 ml of 50% TCA were added. At that time, the sample taken from the first incubation was correspondingly diluted with 5% TCA. Both samples were then prepared for counting by the method described in Table 1.

The quantity of phosphate fixed by the enzyme is dependent upon total phosphate concentration but independent of the amount of radioactive phosphate present (Table 3), indicating that it is indeed the phosphate that is incorporated into the enzyme and not a radioactive contaminant. Moreover, arsenate inhibits incorporation of the phosphate (Table 4).

TABLE 3

DEPENDENCE OF PHOSPHATE FIXATION ON CONCENTRATION

Phosphate added		Phosphate incorporated	
Conc. μM/ml	cpm/μM	cpm/9.4 mg enzyme	Moles phosphate/ moles enzyme
0.1	2.2	355	0.009
1	0.44	260	0.035
10	0.11	415	0.22

Incubation was at 30° for 6 min in 0.2 *M* Tris buffer pH 8, and 0.001 *M* $MgSO_4$, in a total volume of 2 ml, each sample containing 9.4 mg of enzyme. The protein was then precipitated by addition of 0.2 ml of 50% TCA and the precipitate washed twice with 5% TCA. The samples were dissolved in 67% acetic acid and aliquots were counted. The remainder was electrophorized at 3 kv for 2 hr in the pH 3.3 buffer system (see legend to Fig. 1). Electrophoresis showed that less than 10% of the radioactivity in the samples separated from the protein as free phosphate; no correction was made for this small contamination. Corresponding values were obtained in other experiments when serine phosphate was measured after hydrolysis of the samples, if the measurements were corrected for the destruction of serine phosphate during hydrolysis by multiplying them by a factor of 4.[2]

The phosphorylation of phosphatase by inorganic phosphate appears to be specific. Serine phosphate was not found in acid hydrolysates of phosphate-treated α-chymotrypsin (Worthington), ribonuclease A (gift of Dr. A. M. Crestfield), or bovine serum albumin (Armour). The peculiarity of the phosphorylation reaction to alkaline phosphatase is nicely confirmed by the observation that an extract from a mutant of *E. coli* (C_4P^-), similar in every respect to the strain, C_4P^+, from which our phosphatase was purified but lacking the ability to make phosphatase, did not fix phosphate. Furthermore, the extract from the P^- mutant was fractionated in the manner used for enzyme purification and none of the resulting fractions fixed phosphate.

Experiments kindly analyzed by Dr. D. E. Koshland, Jr., showed that during incubation with inorganic phosphate in water containing O^{18}, only a slow exchange of O^{18} into the phosphate occurred. The quantity exchanged was one-tenth of the

amount of O^{18} incorporated into the phosphate produced during the complete enzymatic hydrolysis of β-glycerol phosphate in H_2O^{18}, which, in turn, was slightly more than one-fourth replacement of O^{16} by the heavy isotope (Table 5). While

TABLE 4

EFFECT OF ARSENATE ON THE INCORPORATION OF PHOSPHATE

Addition	cpm/8 mg enzyme
None	5,466
Sodium arsenate, 0.025 *M*	714

Eight mg of enzyme were incubated for 5 min at 30° in 0.2 *M* Tris HCl buffer pH 8, with 0.001 *M* phosphate specific activity 3×10^6 cpm/μM. The samples were prepared for counting as described in Table 1.

TABLE 5

INCUBATIONS IN H_2O^{18}

Addition	O^{18} atoms % excess in isolated inorganic phosphate
β-glycerol phosphate	0.367; 0.38
Inorganic phosphate	0.036

Incubation in both cases was at 30° for 17 hr in 30 ml of water with 1.5% excess H_2O^{18} (Stuart Oxygen Co.), buffered at pH 8 by 0.1 *M* Tris HCl. All reagents were added in dry form, except enzyme, which was added to each incubation in 0.3 ml containing 0.15 mg, and a small amount of concentrated HCl to adjust pH. In one incubation, 0.025 *M* β-glycerol phosphate was present, in the other, 0.025 *M* inorganic phosphate. The quantity of enzyme used at its initial rate will completely hydrolyze the amount of substrate in the experiment in 165 min. After incubation, phosphate was isolated and purified by a modification of the method of Jones and Spector.[19]

these results are consonant with previous studies on alkaline phosphatases from other sources,[20] the slowness of the O^{18} exchange is puzzling when one considers the characteristics of phosphate fixation by the enzyme.

Comments.—The present observations with phosphatase remind us of an earlier-observed reaction of liver or pancreatic esterases[21] with fatty acids to give, in the presence of hydroxylamine, the corresponding hydroxamates; this suggested an activation of the carboxyl group, presumably by a covalent binding of the carboxylate by the esterase. Analogously, phosphatase is now shown to fix inorganic phosphate. The results reported here, which are essentially in accordance with those obtained by Ågren and Engström, pose questions on the energetics of the phosphate binding, some of which we will try to formulate without, however, attempting to give any definite answers. Our dilution experiments indicate an easily reversible link between the phosphate and the enzyme, yet O^{18} exchange with phosphate is very slow. If, furthermore, one considers the relatively high group potential of the phosphoryl group in serine phosphate which, in the classical phosphoprotein, actually approaches that of the terminal phosphate in ATP,[6] incorporation of 1 mole of phosphate to 1 mole of enzyme with only a ten-fold excess concentration of phosphate is difficult to understand. Nevertheless, a strong affinity for phosphate had been deduced earlier from the inhibitory effect of inorganic phosphate at quite low concentration[9] as well as from equilibrium dialysis experiments by Levinthal and his associates.[22] One might then think of the possibility of a transesterification where phosphate displaces a previous ligand to the hydroxyl group. It has indeed been proposed that the hydroxyl group of serine at the active center of esterases is esterified with the free carboxyl group of a neighboring dicarboxylic amino acid,[23] or is fused in an oxazoline ring.[24] The possibility that phosphate fixation may be an exchange with phosphate already

bound to serine seems to be eliminated by the observation that the enzyme, as purified here, does not contain sufficient phosphate to account for an exchange, and that serine phosphate is not found in amino acid analysis.[22]

We hope that some of the questions raised here may find an answer when the amino acid sequence around the reactive serine has become known. So far, we believe the indications are strong that this phosphate binding site participates in the catalytic function of the enzyme.

Summary.—Highly purified *E. coli* alkaline phosphate reacts with inorganic phosphate yielding serine phosphate and serine phosphate-containing peptides on partial acid hydrolysis. After exhaustive digestion of the enzyme with trypsin, only one major component appears containing radioactivity. This product may be degraded by acid hydrolysis to serine phosphate and the same peptides obtained from hydrolysis of the whole enzyme. It is concluded that of the 33 serine molecules known to be present in this phosphatase, only a particular one reacts with the phosphate and that the phosphate-binding serine most likely is in the active center of the enzyme.

Note added in proof: The following experiment appears to show conclusively that the serine accepting the phosphate is in the catalytically active center. Three samples of 0.011 μmole of enzyme in 0.5 ml Tris buffer, pH 5.5, were incubated at 0° for 15 sec with: (1) P^{32}-phosphate, (2) P^{32}-phosphate + an equal amount of P^{31}-glucose 6-phosphate, and (3) P^{32}-phosphate + an equal amount of fully hydrolyzed P^{31}-glucose 6-phosphate. Reaction was stopped by the addition of 0.1 ml of 2 *N* HCl, following the method of Engström;[8] the protein was collected by precipitation with acidified acetone, hydrolyzed at 100° for 19.5 hr with 2 *N* HCl, and electrophorized as described. The radioactivity of the serine phosphate fraction was cut out and counted directly. The following values (not corrected for serine phosphate hydrolysis) were obtained: (1) P^{32}-phosphate alone, 4,412 cpm, (2) P^{32}-phosphate + equal amount of cold phosphate ester, 472 cpm, (3) control, P^{32}-phosphate + equal amount of cold phosphate, 2,120 cpm. The experiment shows that substrate phosphate competes favorably with inorganic phosphate for the specific serine in the enzyme.

The authors wish to note with gratitude the help, encouragement, and advice of C. Levinthal and his group. Many of the methods used here were learned in Dr. Levinthal's laboratory, and we should like especially to thank F. Rothman with whom some of the early trypsin experiments were performed, and A. Garen and A. Torriani from whom the constitutive strains were obtained. Furthermore, we are indebted to L. Engström for generous information about his experiments with *E. coli* enzyme prior to publication.

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† Abbreviation: TCA, trichloroacetic acid.

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A GENETIC LOCUS FOR THE REGULATION OF RIBONUCLEIC ACID SYNTHESIS

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More than 80 per cent of the total ribonucleic acid (RNA) of bacteria is contained in *ribosomes*, the particles which appear to be the site of protein synthesis in the cell, and most of the remainder is accounted for by the "soluble" acceptor RNA involved in the activation of amino acids for protein synthesis.[1-3] A very minor fraction of the bacterial RNA is represented by the ephemeral *messenger* molecules, which, according to recent hypotheses on the mechanism of protein synthesis, are the primary gene products of the cistrons of the bacterial deoxyribonucleic acid that harbor the sequence information for the ordered copolymerization of amino acids into specific polypeptides.[4-6] Whereas the formation of messenger RNA, and hence of the *quality* of protein synthesis, is controlled by various metabolites that act as either inducers or repressors of the functional expression of specific parts of the bacterial genome,[4] the formation of ribosomal RNA, and hence of the *quantity* of protein synthesis, seems to depend on the availability of amino acids. Thus, auxotrophic bacterial mutants requiring an amino acid for growth stop synthesizing not only protein but also bulk, and hence ribosomal,