Alkaline Phosphatase of *Escherichia coli*. Composition*

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ABSTRACT: A rapid, single-step purification of the alkaline phosphatase of *Escherichia coli* using salt gradient chromatograpy on DEAE-cellulose is described. The product is homogeneous by electrophoretic and ultracentrifugal criteria and its amino acid composition is documented. The specific activity of the resultant enzyme is equivalent to that of the crystalline material (Malamy, M. H., and Horecker, B. L. (1964), *Biochemistry 3*, 1889).

Phosphatase prepared in the fashion described contains 2750 μg of Zn/g, nearly 4 g-atoms of Zn/mol wt 89,000, and significant amounts of magnesium and iron. In contrast, the zinc content of phosphatase prepared by other methods previously available is lower than that of material obtained in the manner here described. Possible causes for the variation and significance of the metal content to the structure and function of the enzyme are discussed.

Transition metal ions serve as essential components of a variety of enzymes (Vallee and Wacker, 1968), including the alkaline phosphatase of *Escherichia coli* (Plocke and Vallee, 1962; Plocke *et al.*, 1962). Previous metal analyses on preparations of this enzyme which are of unknown homogeneity and variable specific activity indicated an average zinc content of 1700 μg/g of protein, ranging from 1100 to 2300 μg per g (Plocke *et al.*, 1962). In the course of organic chemical modifications of alkaline phosphatase in this laboratory, enzyme, isolated by a method proposed more recently (Malamy and Horecker, 1964b), was found to contain more zinc than most preparations previously examined by us (Tait and Vallee, 1966). Thus, while the essentiality of zinc for the activity of phosphatase is established, the stoichiometry of metal to protein seemed variable. Hence, a study of homogeneity of alkaline phosphatase, its molecular weight, its metal composition, and the relationship, if any, of the latter to the method of preparation was undertaken, prior to more detailed investigations of the role of metal ions in structure and function of the enzyme.

The chromatographic preparation of phosphatase here employed leads in a single step to protein which is homogeneous by electrophoretic and sedimentation criteria. Its amino acid composition has been established and its metal content has been determined on a number of preparations at various stages of purification. Spectrographic analyses demonstrate the presence of 2750 μg of Zn/g, nearly 4 g-atoms of Zn/mole of protein, employing a molecular weight of 89,000 as determined on these preparations (R. T. Simpson, J. L. Bethune, and B. L. Vallee, in preparation). In contrast, enzyme prepared by crystallization from $(NH_4)_2SO_4$ (Malamy and Horecker, 1964b) contains approximately 3 g-atoms of zinc, assuming the same molecular weight.

Experimental Section

E. coli, strain C-90, was grown to the stationary phase at 37° in a medium of 0.12 M Tris-Cl, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 0.03 M Na_2SO_4, 0.001 M $MgCl_2$, 2×10^{-4} M $CaCl_2$, 5×10^{-6} M $ZnSO_4$, and 0.5% Difco proteose–peptone no. 3, adjusted to pH 7.4 with HCl. Dextrose was autoclaved separately and added at the time of inoculation to achieve a final concentration of 0.5%. Initially, growth was carried out in total culture volumes of 6–12 l. Later preparations were grown in a 60-l. fermenter at the New England Enzyme Center.

The organisms were harvested by centrifugation in a Sharples continuous-flow centrifuge and washed twice with a volume of 0.01 M Tris-Cl buffer (pH 7.5) equivalent to 10% of the original culture volume. The washed organisms were subjected to osmotic shock (Neu and Heppel, 1965), using 5% of the initial culture volume both of the 20% sucrose, 1×10^{-3} M EDTA, 0.03 M Tris-Cl buffer and cold, distilled water. The water extract thus obtained constituted the starting material for the chromatographic separation described. For comparison, phosphatase was also prepared by step elution from DEAE-cellulose and crystallization from $(NH_4)_2SO_4$ (Malamy and Horecker, 1964b), using as starting material either the water extracts obtained by osmotic shock (Neu and Heppel, 1965) or the solution obtained by lysozyme treatment (Malamy and Horecker, 1964a).

DEAE-cellulose (DE-23, Whatman Co.) was cleaned as described by Peterson and Chiazze (1962) and adjusted to pH 7.2 with dilute HCl. The absorbant was

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then equilibrated with either 0.01 M Tris-Cl or 0.01 M imidazole-Cl (pH 7.2). The cellulose was packed into columns using steadily increasing pressures of nitrogen to a final pressure of 15 psi. After packing, the columns were washed with three to six column volumes of starting buffer prior to absorption of the protein solution.

Emission spectrographic metal analyses (Vallee, 1955) were performed on all fractions of the various preparations. Zinc and magnesium were also determined by atomic absorption spectrometry (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964; Iida *et al.*, 1967). The zinc content of three of the enzyme preparations was confirmed by means of a microchemical measurement using the dithizone method (Vallee and Gibson, 1948). Prior to metal analyses, all samples were dialyzed exhaustively (*vide infra*) against 0.01 M Tris-Cl buffer (pH 7.5). A buffer "blank" was carried through all determinations, and at no time could zinc be detected in this "blank."

Amino acid analyses were performed chromatographically with a Beckman 120B automatic amino acid analyzer according to Spackman *et al.* (1958). Hydrolysis was carried out for varying times in sealed, evacuated tubes with constant-boiling HCl. Phenol (5 μl) was added to each hydrolysis tube in order to prevent halogenation of tyrosine, and norleucine was added as an internal standard. Tryptophan and cystine were determined colorimetrically with dimethylaminobenzaldehyde (Spies and Chambers, 1949) and fluorescein mercuric acetate (Karush *et al.*, 1964), respectively.

Amino acid analyses and metal contents are expressed on a molecular weight for phosphatase of 89,000 (R. T. Simpson, J. L. Bethune, and B. L. Vallee, in preparation). A value of 0.72 was employed as the absorbance at 278 mμ of a 1-mg/ml solution of the enzyme (Plocke *et al.*, 1962). This extinction coefficient was confirmed by measurement of protein dry weight following precipitation with trichloroacetic acid (Hoch and Vallee, 1953).

Disc gel electrophoresis was performed at pH 9.4 using the conditions suggested (Canalco Corp.). Moving-boundary electrophoresis was carried out in the Spinco Model H apparatus at a protein concentration of 6 mg/ml in 0.01 M Tris-Cl–0.1 M KCl (pH 7.0), at a gradient of 1.32 V/cm and a temperature of 1°. Sedimentation velocity measurements were obtained in the Spinco Model E analytical ultracentrifuge equipped with an RTIC unit and a phase plate as schlieren diaphragm. Centrifugation was performed at 22° with 12 mg of protein/ml of 0.01 M Tris-Cl–0.1 M KCl (pH 7.0).

Enzymatic activity was assayed by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 400 mμ. The reaction was initiated by addition of enzyme to yield a final concentration of about 1 μg/ml in 3 ml of 1×10^{-3} M *p*-nitrophenyl phosphate in 1 M Tris-Cl (pH 8.0). The change in absorbance was followed for a period of at least 1 min using a Unicam SP 800 recording spectrophotometer, thermostatted at 25°. Specific activities refer to the hydrolysis of 1 μmole of substrate/min per mg of protein under these conditions, based upon a molar absorbancy of *p*-nitrophenol in 1 M Tris-Cl (pH 8.0) of 1.7×10^4 M^{-1} cm^{-1}.

Measurements of absorbance at discrete wavelengths were carried out in a Zeiss PMQ II spectrophotometer A Radiometer pH meter with a general-purpose glass electrode was utilized for determination of pH, while a Radiometer conductivity meter with a CDC 114 electrode was employed for the measurement of conductivity during chromatographic experiments.

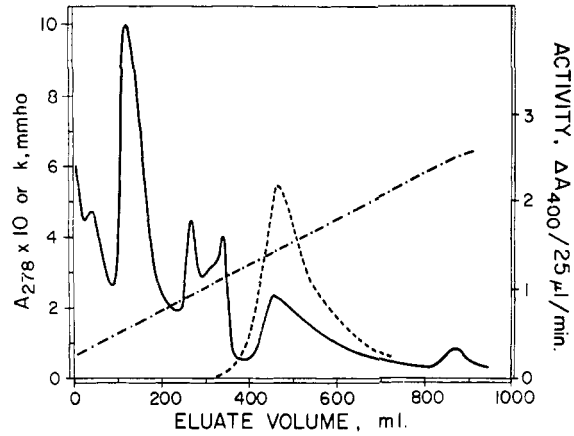

FIGURE 1: DEAE-cellulose chromatogram of the water extract of a 3-l. culture of *E. coli*. The water extract was adjusted to contain 0.01 M imidazole-C (pH 7.1) and applied to a 0.9 × 25 cm column of DEAE-cellulose prepared as in the Experimental Section. Gradient elution was carried out with 500 ml each of the starting and limit buffers at room temperature. Conductivity (—·—·—·—) absorbancy at 278 mμ (—) and enzymatic activity (– – – – – –) are plotted.

Precautions were taken to avoid metal ion contamination during the growth of organisms and the purification of the protein (Thiers, 1957). All glassware was cleaned with acid and deionized, distilled water. Prior to metal analyses all samples were dialyzed against three to four changes of 50–100-fold volume excesses of metal-free buffers for at least 8 hr each. These solutions were prepared by dilution of 2 M Tris-Cl (pH 8.0), freed of trace metal contaminants by passage over a Chelex 100 (Bio-Rad Corp.) column (Himmelhoch *et al.*, 1966). Buffers prepared in such fashion were free of detectable contamination with zinc, either measured directly or after ashing of the organic components.

Purification of Phosphatase. The water extract derived from osmotic shock of the washed organisms is adjusted to contain 0.01 M imidazole-Cl (pH 7.2) (the starting buffer). The sample is then applied to a DEAE-cellulose column equilibrated with starting buffer, using 1 g of DEAE-cellulose (dry weight)/l. of original culture volume. Following adsorption of the protein, the column is washed with two column volumes of the starting buffer. Gradient elution is then carried out with a linear gradient of equal volumes of starting buffer and starting buffer plus 0.1 M NaCl. Generally, the total gradient volume was 30–40 ml/ml of column volume.

The results of chromatography of the water extract from a 3-l. culture, on a 3-g DEAE-cellulose column, with a 1000-ml gradient are shown in Figure 1. Phosphatase emerges as a distinct peak with a sharp leading edge and a diffuse trailing edge, well separated from three preceding species. The peak contains approximately 80% of the activity applied to the column.

TABLE I: Chromatographic Purification of Alkaline Phosphatase.

Sample	Vol (ml)	Protein (mg/ml)	Enzymatic Act.[a] Total Units	Enzymatic Act.[a] Units/mg	Yield (%)
Culture	6000	*b*	5.2×10^8	*b*	100
Water extract	220	2.4	3.7×10^8	7.0	71
DEAE peak after concentration	10	7.0	3.0×10^8	43.0	58

[a] Determined in 1 M Tris-Cl, as described in the Experimental Section. [b] Not determined.

TABLE II: Metal Content during Purification of Alkaline Phosphatase.[a]

Sample	Zn	Sr	Ba	Fe	Ca	Cr	Mn	Al	Ni	Mo	Mg
Washed cells	350		9	>2500	510	>250	77	88	>770	95	1500
Water extract	840	Tr	14	520	300	10	25	65	Tr	Tr	125
Purified enzyme	2500		5	500	100		45	29	Tr	120	435

[a] All results are the averages of duplicate analyses and are expressed as micrograms of metal per gram of protein. Pb and Cd were not detected in any sample. Tr = trace; a blank space indicates not detected. Cu was not determined due to the method employed (Vallee, 1955).

The peak tubes are pooled and diluted to a conductivity of <1.5 mmhos, and then reapplied to a small column of DEAE, usually 10% of the initial column size. The second column is developed by step elution with starting buffer plus 0.1 M NaCl to yield the purified protein in a conveniently small volume. Alternatively, the pooled peak fractions may be concentrated as desired by pressure dialysis through a UM-1 membrane in an Amicon Corp. dialysis cell. The enzyme is stored at 4° in 1 M NaCl solution, saturated with toluene.

When the organism is grown in large quantities, the enzyme from the correspondingly larger volumes of water extract which results may be absorbed onto DEAE-cellulose in a batchwise fashion, using 30–40 g of absorbent/100 l. of culture volume. The enzyme is then eluted from the cellulose on a Buchner funnel with 0.1 M NaCl in starting buffer, concentrated by pressure dialysis, and dialyzed into starting buffer for gradient chromatography. Since this procedure removes a large amount of functionally inert protein, resulting in a threefold increase of specific activity, the size of the DEAE-cellulose column employed for gradient chromatography can be reduced. A 40-g column with an 8-l. gradient can be employed conveniently for the purification of phosphatase derived from a 100-l. culture of *E. coli*.

Results

The specific activities and yields obtained during a typical purification of phosphatase by the chromatographic procedure described are detailed in Table I. Phosphatase constitutes approximately 15% of the protein content of the water extract, which is a convenient starting material, readily handled for chromatographic separation. A single gradient chromatogram on DEAE-cellulose increases the specific activity of the preparation to a value equivalent to that obtained for the enzyme recrystallized from $(NH_4)_2SO_4$ (Malamy and Horecker, 1964b). The specific activity of phosphatase prepared by this chromatographic method is not increased by crystallization.

The increase in specific activity during purification parallels that of zinc content. Concurrently, the content of most other metals detected by emission spectrography decreases upon proceeding from the original bacterial cells to the final purified protein (Table II). An accumulation of magnesium in the final product with respect to the initial water extract constitutes an exception to this general trend. The iron content of the extract, about 500 μg/g, remains unaltered during chromatography.

Weight homogeneity of such alkaline phosphatase preparations has been examined by two methods, *i.e.*, gel filtration and sedimentation analysis. Both failed to demonstrate the presence of contaminating species. The purified protein elutes from Sephadex G-100 as a single symmetric peak with a specific activity equal to that of the material applied to the column. Further, sedimentation velocity experiments in the analytical ultracentrifuge reveal the presence of a single, symmetric sedimenting boundary with a sedimentation constant of 6.2×10^{-13} sec^{-1} (Figure 2b).

Similarly, these phosphatase preparations are free from detectable contamination with species of differing electrophoretic mobility, both by free-boundary electro-

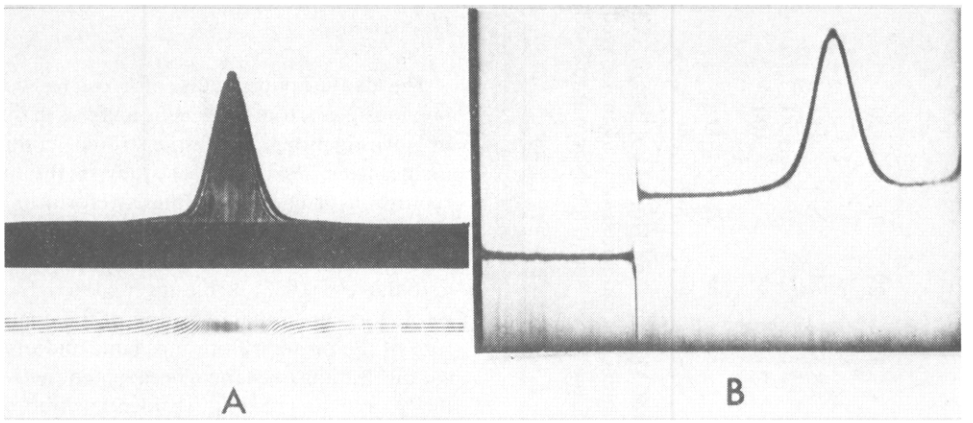

FIGURE 2: Tiselius electrophoresis and ultracentrifugation of alkaline phosphatase. (A) The photograph was taken 1100 min after commencing electrophoresis at a gradient of 1.32 V/cm and a temperature of 1°. The concentration of the protein is 6 mg/ml in 0.01 M Tris-C–0.1 M KCl (pH 7.0). The direction of migration is from right to left. (B) The photograph was taken 60 min after reaching speed (59,780 rpm) at a phase-plate angle at 70°. The protein concentration was 12 mg/ml in 0.01 M Tris-C–0.1 M KCl (pH 7.0). The direction of sedimentation is from left to right.

phoresis at pH 7.0 (Figure 2a) and by disc electrophoresis on polyacrylamide gels at pH 9.4. Heavy loads of protein were applied to the gel columns in attempts to ascertain the possible presence of nonphosphatase contaminants but none were detected. At small total loads of protein, *e.g.*, 5 μg, the purified protein consists of an approximately 95:5 mixture of two species, both of which are enzymatically active, the less anionic protein predominating in amount.

Amino acid analyses were performed in duplicate on four samples of enzyme hydrolyzed for 24 hr, two hydrolyzed for 48 hr, and two for 72 hr. The results of these analyses, together with colorimetric determinations of tryptophan and cystine, are presented in Table III. A time dependence of the yield of the amino acids was observed only for serine and threonine, and the data for these two species are extrapolated to zero time of hydrolysis. In addition to the average values obtained from the 16 analyses, the table includes the standard deviation of the mean analytical value for the individual amino acids as an indication of the precision of the determinations.

The emission spectrographic data obtained for five separate phosphatase preparations are compiled in Table IV. Zinc, the major metallic constituent, varies from 2500 to 3100 μg per g of protein corresponding to 3.5 to 4.2 g-atoms per mole of protein, based on a molecular weight of 89,000, with an average of 3.8. In four of the five chromatographically prepared samples, magnesium is found in amounts corresponding to 1.6–2.0 g-atoms/mole of protein. Iron is the only other metal consistently present in amounts approaching stoichiometric significance, ranging from 400 to 700 μg per g in all samples and corresponding to a content of 0.6–1.0 g-atoms/mole of protein.

Similar spectrographic data on phosphatase prepared in this laboratory by crystallization from $(NH_4)_2SO_4$ and $MgCl_2$ (Malamy and Horecker, 1964b) indicate the presence of approximately 3 g-atoms of zinc/mole of protein and perhaps more magnesium than present in the chromatographic preparations (Table IV). The specific activities of one of these preparations, actually that with the higher zinc content, was slightly lower than those of the enzymes prepared chromatographically, while the other was of the same order. Yet other methods of preparation employed have yielded values for zinc content of about 2.5–3 g-atoms/mole (Harris and Coleman, 1968).

TABLE III: Amino Acid Composition of Alkaline Phosphatase.[a]

Amino Acid	Residues/ Mole	Std Dev[b]	% Std Dev[b]
Lysine	50.3	0.5	1.0
Histidine	16.4	0.1	0.8
Arginine	23.1	0.3	1.3
Aspartic acid	92.5	0.6	0.7
Glutamic acid	88.4	0.7	0.8
Proline	39.4	0.7	1.7
Glycine	86.0	0.9	1.0
Alanine	124.4	1.2	1.0
Valine	43.0	1.0	2.6
Methionine	14.4	0.2	1.1
Isoleucine	27.7	0.3	1.2
Leucine	75.4	0.5	0.7
Tyrosine	20.3	0.2	1.2
Phenylalanine	16.0	0.3	1.6
Serine[c]	76.0		
Threonine[c]	42.0		
Cystine (half)[d]	8.0		
Tryptophan[e]	7.9		

[a] Determined as in Experimental Section; results are the means of 16 analyses except as noted below. [b] Standard deviation of the mean analytical value. [c] Results extrapolated to zero time of hydrolysis. [d] Colorimetric determination with fluorescein mercuric acetate (Karush *et al.*, 1964). [e] Colorimetric determination with dimethylaminobenzaldehyde (Spies and Chambers, 1949).

TABLE IV: Activities and Metal Contents of Purified Alkaline Phosphatase.

Preparation[a]	Sp Act.	Zn		Mg		Fe		Ba	Ca	Cr	Mn	Al	Ni	Mo
		μg/g	g-atoms/Mole	μg/g	g-atoms/Mole	μg/g	g-atoms/Mole	μg/g						
Chrom I	39	2500	3.5	430	1.6	500	0.7	5	100		45	29	Tr	120
Chrom II	43	3100	4.2	Tr		700	1.0	6			33	41	Tr	
Chrom III	40	2800	3.9	560	2.0	ND		7	Tr	ND	135	74	ND	Tr
Chrom IV	39	2600	3.7	480	1.8	500	0.7		150	74	Tr	75		70
Chrom V	42	2700	3.8	520	1.9	400	0.6	Tr	350	Tr	Tr	55		100
Cryst I	36	2200	3.0	760	2.8	570	0.8	6	270	38	28	66	280	Tr
Cryst II	40	2000	2.8	550	2.0	ND		4	75	ND	42	46	ND	Tr

[a] Chrom = enzyme prepared chromatographically (see Experimental Section). Cryst = crystalline enzyme prepared by the method of Malamy and Horecker (1964b). All results are the averages of duplicate analyses. Tr = trace, blank space = not detected, ND = not determined. Activities are expressed as units per milligram of protein, assayed in 1 M Tris-Cl, as described in the Experimental Section. The elements which are not listed were not detected except copper which cannot be measured due to the system employed (Vallee, 1955).

Discussion

The alkaline phosphatase of *E. coli* has been employed previously as a tool for genetic analysis in this bacterium, in investigations of intracistronic complementation (Schlesinger, 1964) and for study of the mechanism of repression of enzyme synthesis (Horiuchi *et al.*, 1959; Torriani, 1960; Echols *et al.*, 1961). More recently, studies of the subunit structure of the protein (Schlesinger and Barrett, 1965; Schlesinger, 1967) have suggested a role for zinc in the formation of the quaternary structure of the protein. Both inorganic and organic modifications leading to changes in enzymatic activity and specificity, and to presumptive identification of one of the zinc binding groups (Tait and Vallee, 1966), have focused attention on the interaction of the metal with the protein. Modifications of the procedures for isolation and crystallization of the enzyme (Malamy and Horecker, 1964a,b; Neu and Heppel, 1965), moreover, suggested the need for an examination of the effect of these procedures on protein structure and metal content.

The role of metals, especially magnesium and zinc, in the mode of action of phosphatase had long been suspected (Cloetens, 1942; Roche, 1946). More recently, zinc was shown to be a catalytically essential component of bacterial (Plocke *et al.*, 1962) and of several mammalian phosphatases (Mathies, 1958; Engstrom, 1961; Trubowitz *et al.*, 1961).

Both of the procedures for the preparation of alkaline phosphatase previously available involved the addition of relatively high concentrations of magnesium ions at one stage of the preparative scheme (Garen and Levinthal, 1960; Malamy and Horecker, 1964b). Conceivably, addition of 0.01 M magnesium might affect the content of intrinsic metal, present in the native enzyme, *i.e.*, by displacing zinc. Ammonia is capable of forming zinc complexes of high stability (Sillen and Martell, 1964). When the enzyme is crystallized from $(NH_4)_2SO_4$, of necessity substantial concentrations of ammonia are present. Conceivably this circumstance could lead to the removal of zinc from the native enzyme. Hence, the present work successfully aimed to prepare phosphatase while avoiding exposure both to magnesium and $(NH_4)_2SO_4$. While the present data suggest that addition of ammonium sulfate and of magnesium may alter the zinc content of the enzyme, this correlation does not necessarily imply that yet other procedural differences, *e.g.*, conditions for growth of the organism, heat denaturation, or lyophilization of the enzyme, might not also affect its metal content.

The water extract obtained from osmotic shock of *E. coli* (Neu and Heppel, 1965) provides a highly purified and easily manageable starting material, and the high resolving power of chromatography on DEAE-cellulose has enabled a rapid, single-step purification of phosphatase. The procedure reproducibly yields alkaline phosphatase of high and constant specific activity in good over-all yield. The specific activity of enzyme prepared chromatographically is comparable with that obtained for crystalline alkaline phosphatase by Malamy and Horecker (1964b). The specific activity of the

chromatographically prepared enzyme is not increased further by such crystallization.

The protein, thus prepared, is homogeneous by a number of physicochemical criteria. Both sedimentation velocity experiments and molecular sieve chromatography fail to reveal the presence of contaminating material of differing molecular size. Similarly, both free-boundary electrophoresis at pH 7.0 and disc electrophoresis at pH 9.4 demonstrate the homogeneity of the protein by these criteria.

While enzymatically inactive material is absent, as judged by disc electrophoresis, two of the "isozymes" (Levinthal *et al.*, 1962) of phosphatase can be detected. This isoenzyme distribution is also apparent in the DEAE-cellulose chromatogram; the emerging protein has a sharp leading edge, but trails off slowly, with a small shoulder on the descending limb of the eluting peak. While chromatography with this particular DEAE-cellulose (DE-23, Whatman Co.) partially resolves the isozymic species, chromatography on a different cellulose (DE-32, Whatman Co.) has allowed separation of the phosphatase "isozymes," permitting their individual investigation (R. T. Simpson and B. L. Vallee, unpublished observations).

The amino acid composition of alkaline phosphatase prepared by this method has been established (Table III) and is nearly identical with previously published preliminary analyses of phosphatase, prepared by other methods (Rothman and Byrne, 1963). For most residues the nearest integral is an even number consistent with the known existence of identical subunits in this protein (Rothman and Byrne, 1963). The absence of cysteine and the relatively low content of the aromatic amino acids are in accord with the findings of other investigations on amino acid composition of *E. coli* phosphatase (Rothman and Byrne, 1963; Schlesinger and Barrett, 1965).

The metal content of enzymatically active fractions is of considerable interest. The content of all metals other than zinc, iron, and magnesium decreases during purification to become negligible in the final product. The increase of zinc during the purification parallels the increase in enzymatic activity at the two stages of the fractionation. The magnesium content of the protein tends to increase from the water extract to the final purified material. Such increases in magnesium had been noted previously (Plocke *et al.*, 1962), but were observed at a stage where magnesium was added to stabilize the protein. Magnesium has long been known to protect the enzyme from heat denaturation (Garen and Levinthal, 1960) and to increase enzymatic activity at low ionic strengths (Plocke and Vallee, 1962). The present data suggest that intrinsic magnesium could be present in sufficient concentration to account for these roles.

The catalytic significance of the magnesium content of the protein is uncertain, however. Previous experiments indicated that addition of magnesium to the zinc-free apoenzyme reconstituted with zinc did not further affect activity (Plocke *et al.*, 1962). Recent studies have demonstrated that magnesium does not form an active enzyme *per se*, although addition of magnesium increases the rate of re-formation of active enzyme from apophosphatase and zinc (K. Fuwa, R. T. Simpson, and B. L. Vallee, unpublished). Molecular size and stability of these reconstituted enzymes are currently under investigation in our examination of the possible role of magnesium in *E. coli* phosphatase.

It should be noted that all of the preparations contained substantial concentrations of iron (Tables II and IV). The functional significance of the presence of this metal is unknown. Thus far, no evidence has been obtained that this element can substitute for zinc either partially or completely.

All the chromatographically purified preparations of phosphatase investigated so far contain nearly 4 g-atoms of zinc/mole of protein (Table IV). In previous analyses of the metal content of the enzyme prepared by other methods, the average zinc content of five preparations, varying in specific activity, was 2.1 g-atoms/mole, when referred to a molecular weight of 80,000. This value increases to 2.3 g-atoms/mole when based on a molecular weight of 89,000. However, the specific activities of preparations which contained less than 2 g-atoms of zinc were lower, while those of two preparations, containing 2.0 and 2.8 g-atoms per 80,000 molecular weight, respectively, were correspondingly higher (2.2 and 3.1 g-atoms based on a molecular weight of 89,000). The series of preparations of phosphatase analyzed in the present investigation do not vary so widely either in specific activity or in metal content.

The average zinc content of these five preparations prepared chromatographically is 3.8 g-atoms/mole of 89,000 molecular weight. It should be noted that accurate definition of the molar stoichiometry of gram-atoms of metal per mole of protein requires not only precise measurement of metal content, but also equally accurate knowledge of protein concentrations and molecular weights. A combined error of about 5% in the measurements of any of these parameters would decrease a hypothetical, integral value of 4 g-atoms of metal/mole to that determined, *i.e.*, 3.8. While the enzyme prepared chromatographically appears to contain 4 g-atoms of zinc/mole of protein, the metal content is lowered when the enzyme is isolated by procedures which involve exposure to high concentrations of ammonia and magnesium during the crystallization (Table IV). This decrease in metal content is not attended either by a significant decrease or increase in enzymatic activity.[1] Similarly, in preparations analyzed previously, one set of analyses indicated that enzyme exhibiting full activity but containing 2 g-atoms of zinc/mole could be prepared (Plocke *et al.*, 1962).

Thus, apparently enzymes of equivalent specific activity can be prepared containing from 2 to 4 g-atoms of zinc per mole of protein. The development of various methods of isolation and characterization of the resulting enzymes has led to the realization that the relation-

[1] The enzyme prepared chromatographically and that prepared by crystallization from $(NH_4)_2SO_4$ also differ in some other respects. Thus the enzyme prepared by chromatography is significantly more stable on storage and against heat denaturation. At least some of the instability on storage seems to be due to loss of metal; excess zinc partially restores activity.

ship of the metal atoms to the structure and function of alkaline phosphatase is more complex than could be recognized previously.

Alkaline phosphatase is thought to consist of identical subunits (Rothman and Byrne, 1963) and is presumed to have two active sites (Levinthal *et al.*, 1962). It would seem plausible that two of the zinc atoms are located at the active sites and participate in substrate binding, catalysis, or both. In this case the presence of less than 2 g-atoms of zinc/mole of protein should lower the specific activity, as has been observed (Plocke *et al.*, 1962). If these two zinc atoms are those more firmly bound, conditions might exist which could enable selective removal of all metals other than those concerned directly with activity.

Avoidance of such conditions during the preparations of the enzyme results in protein containing nearly 4 g-atoms of zinc/mole. These additional zinc atoms may well stabilize the tertiary or quaternary structure of the protein, similar to zinc in horse liver alcohol dehydrogenase (Drum *et al.*, 1967), yeast alcohol dehydrogenase (Kägi and Vallee, 1960), and α-amylase of *Bacillus subtilis* (Vallee *et al.*, 1959; Stein and Fischer, 1960). Evidence supporting this interpretation is the subject of other communications (R. T. Simpson, J. L. Bethune, and B. L. Vallee, in preparation).

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