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Zinc and Magnesium Content of Alkaline Phosphatase from *Escherichia coli*†

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ABSTRACT: Since alkaline phosphatase from *Escherichia coli* was first reported to contain 2.1 g-atoms of zinc and 0.8 g-atom of magnesium per molecular weight 80,000 (Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1,* 373–378), the procedures for isolation and purification of the enzyme, as well as values for the protein molecular weight, specific absorptivity, and maximal activity, have changed repeatedly. Such variations have resulted in uncertainties concerning the molar metal content of this phosphatase. The present paper reviews the initial and recent results of metal analyses of alkaline phosphatase preparations in this laboratory and compares them with those obtained elsewhere, while simultaneously identifying some of the factors which have affected reports on the metal content of this enzyme. A purification procedure is described eliminating the features of all methods known to alter the metal content of phosphatase. In addition, the three isozymic forms, as well as preparations from four *E. coli* strains commonly employed for phosphatase isolation, were analyzed and compared. Collectively, the zinc content was found to be 4.0 ± 0.3 g-atoms per molecular weight of 89,000 for enzyme purified and analyzed by procedures shown not to alter its intrinsic metal content adversely. Importantly, it is confirmed that the enzyme when isolated at pH 7.2 and 7.5 contains an average of 1.3 ± 0.2 g-atoms of magnesium/mol. This feature of the metal composition, hitherto largely unappreciated, bears significantly on the investigation of the structure of alkaline phosphatase as related to its chemical and functional properties.

The precision and sensitivity of methods applicable to the determination of the physical, chemical, and functional properties of enzymes have increased markedly in the last decade. Concurrently, there have been analogous advances in high resolution procedures for enzyme isolation. Purification of enzymes by means of such superior methodology may result in numerical values for the parameters characterizing their properties which differ from those obtained initially. Unfortunately, such determinations frequently do not indicate definitively whether the numerical changes observed result from intrinsic differences in the enzyme being examined, variations in analytical methodology, or differing modes of calculation and representation of results.

Escherichia coli alkaline phosphatase well illustrates these evolving problems in protein characterization. It was first isolated in 1960 (Garen and Levinthal, 1960) and soon thereafter shown to contain stoichiometric quantities of zinc and magnesium (Plocke et al., 1962). Since these early studies, native phosphatase, prepared from *E. coli* by a variety of methods, consistently has been shown to require minimally 2 g-atoms of zinc/mol of enzyme for catalytic activity (Simpson et al., 1968; Harris and Coleman, 1968; Reynolds and Schlesinger, 1969; Petitclerc et al., 1970; Trotman and Greenwood, 1971; Csopak and Szajn, 1973). Further, it was demonstrated that two additional zinc atoms stabilize secondary, tertiary, or quaternary structure and/or

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influence catalytic activity (Simpson et al., 1968; Reynolds and Schlesinger, 1969; Petitclerc et al., 1970; Trotman and Greenwood, 1971). However, the presence and the functional significance of these two additional zinc atoms have incurred considerable debate (Harris and Coleman, 1968; Applebury and Coleman, 1969; Csopak et al., 1972a; Csopak and Szajn, 1973).

Even though magnesium has been reported to enhance activity of the zinc enzyme (Plocke and Vallee, 1962), virtually all workers have examined only the stoichiometry of zinc in their preparations, while that of magnesium consistently has been ignored, and systematic studies of its possible role in catalysis and/or structure stabilization are not on record. Furthermore, as a general rule phosphatase metal content has been expressed in terms of g-atom of metal per mole of protein rather than as the ratio of mass of metal per g of protein. Importantly, the molecular weight of the enzyme upon which such molar metal stoichiometry is based has varied significantly (Garen and Levinthal, 1960; Schlesinger and Barrett, 1965; Simpson et al., 1968; Applebury and Coleman, 1969; Trotman and Greenwood, 1971). Similarly, the absorbance at 278 nm (Plocke et al., 1962; Rothman and Byrne, 1963; Malamy and Horecker, 1964; Csopak et al., 1972b) and maximal specific activity (Plocke et al., 1962; Lazdunski and Lazdunski, 1967; Simpson et al., 1968; Harris and Coleman, 1968; Reynolds and Schlesinger, 1969; Csopak and Szajn, 1973) have differed widely. A number of factors may affect the value of these parameters: (1) the strain of *E. coli* employed, (2) the ambient conditions used in releasing phosphatase from the organism, (3) the procedures for subsequent purification, and (4) the analytical methodology and calculations used to define the characteristics of the enzyme.

Initially, phosphatase was isolated from whole cell extracts and purified by precipitation at 80° in high concentrations of magnesium followed by chromatography on DEAE-cellulose (Garen and Levinthal, 1960; Plocke et al., 1962). Since then, phosphatase purification procedures have been modified and improved repeatedly. Thus, following the finding that phosphatase is localized in the periplasmic space (Malamy and Horecker, 1961), the enzyme was released from *E. coli* by a variety of techniques, including lysozyme treatment in the presence of EDTA (Malamy and Horecker, 1964), osmotic shock in the presence of chelating agents (Neu and Heppel, 1965), and treatment of cells at acid pH releasing metal-free subunits requiring activation (Schlesinger and Olsen, 1970). Following release, the enzyme generally was purified by means of DEAE-cellulose chromatography, gel filtration, and ammonium sulfate precipitation or crystallization. The purified enzyme, however, can be resolved into three forms by high resolution DEAE-cellulose chromatography, although the characteristics of these isozymes have not been examined in detail (Lazdunski and Lazdunski, 1967; Simpson et al., 1968). Several of the aforementioned procedures, viz., exposure to EDTA or ammonium sulfate and addition of high concentrations of magnesium and/or zinc, previously have been suggested as parameters responsible for altering the metal content of native enzyme (Simpson et al., 1968; Ulmer and Vallee, 1971; Csopak and Szajn, 1973).

The present paper summarizes the metal content of the consecutively numbered alkaline phosphatase preparations since such studies were first initiated in this laboratory. They are compared with analogous data on preparations obtained elsewhere using various methods of purification. The study confirms the presence of zinc and magnesium in alkaline phosphatase from *E. coli*, delineates their stoichiometry, and simultaneously identifies some of the factors which may affect the metal content of this enzyme. Further, a purification procedure is described which eliminates the features of all methods known to alter metal content of phosphatase. In addition, it is emphasized that careful observance of metal-free technique (Thiers, 1957) and ashing of alkaline phosphatase samples prior to analysis by atomic absorption spectrometry are essential to accurate determination of the enzyme's metal content. Collectively, the data demonstrate that the phosphatase contains 4.0 ± 0.3 g-atoms of zinc and 1.3 ± 0.2 g-atoms of magnesium per molecular weight 89,000 when isolated and purified at pH 7.2 and 7.5 by methods which do not affect its metal content adversely.

Experimental Section

Purification of Alkaline Phosphatase. In this laboratory preparations of alkaline phosphatase were initially obtained from cultures of *E. coli* K-12 either wild-type or constitutive for phosphatase, grown in a lactate–inorganic salts or tryptone–yeast extract medium (Table II, no. 1–5) (Plocke et al., 1962). Acetone powders from whole cell extracts were then purified utilizing heat precipitation at 80° in 0.01 *M* $MgSO_4$ followed by chromatography on DEAE-cellulose.

Preparations 6–26, Tables II–IV, were obtained from periplasmic extracts of *E. coli*. Strain C-90 (Tables II and III, no. 6–20) was grown to stationary phase at 37° in 100- or 14-l. cultures in a Tris-peptone medium (Simpson et al., 1968), and strains K-10, C_4F_1, and CW-3747[1] (Table IV, no. 21–26) were grown similarly in a 3-l. culture using "121 Peptone" medium (Torriani, 1966). The cells were harvested by centrifugation and washed in 0.01 *M* Tris-Cl (pH 7.5). Periplasmic extracts were then prepared using osmotic shock *either* in sucrose and EDTA *or* sucrose and Chelex 100 (Neu and Heppel, 1965). When EDTA was utilized to release the enzyme (Tables II–IV, no. 6–16 and 21–26) a 2–6% cell suspension was prepared at room temperature in 20% sucrose, 10^{-3} *M* EDTA, and 0.03 *M* Tris-Cl (pH 7.5), and followed by centrifugation. The cells were shocked with the same volume of cold, distilled water. When Chelex 100 (Bio-Rad, 1–200 mesh) was used (Table III, no. 17–20), a 2% cell suspension was prepared in 5% Chelex, 20% sucrose, and 0.03 *M* Tris-Cl (pH 7.5). Under these circumstances, an additional centrifugation for 5 min at 600*g* was required to separate cells from Chelex, thereby assuring that the phosphatase released subsequently never comes into contact with any chelating agent and eliminating all effects in this step potentially attributable to EDTA (Ulmer and Vallee, 1971; Csopak and Szajn, 1973).

The water extract from the osmotic shock medium was purified by batchwise absorption on DEAE-cellulose (Whatman DE52), equilibrated with 0.01 *M* imidazole-Cl (pH 7.2), and eluted with 0.1 *M* NaCl. The protein was concentrated and dialyzed by ultrafiltration (Amicon PM-10 membrane).

Preparations 6–26, Tables II–IV, were purified by column chromatography on DEAE-cellulose in 0.01 *M* imidazole-Cl (pH 7.2), using a linear gradient of NaCl to 0.1 *M*. The broad peak of activity, containing the isozymes, was

[1] Kindly provided by Dr. A. M. Torriani, Department of Biology, M.I.T.

concentrated and dialyzed by ultrafiltration. In some cases rechromatography on DEAE-cellulose (Table III, no. 15 and 19) or on Sephadex G-150 (Pharmacia, 40–120 μ) in 0.01 *M* Tris-Cl (pH 7.5) (Table III, 17 and 18) was employed to obtain enzyme of high specific activity. Preparations 6 and 7, Table II, were prepared by Simpson et al. (1968) using crystallization from $(NH_4)_2SO_4$ and $MgCl_2$ according to the method of Malamy and Horecker (1964).

Phosphatase Assays. Enzymatic activity was measured by following the hydrolysis of 4-nitrophenyl phosphate (Sigma 104) using a Gilford Model 222 spectrophotometer with a Heathkit Model IR-18M recorder. The release of 4-nitrophenol in 1 *M* Tris-Cl (pH 8.0) and 25° was determined at 400 nm using a molar absorbance of 1.68×10^4. A unit of activity is defined as the number of micromoles of substrate hydrolyzed per minute per milligram of protein under these conditions. Phosphatase concentration was determined spectrophotometrically using $A_{278}(1\%)$ 7.2 (Simpson et al., 1968).

Electrophoresis. Polyacrylamide disc gel electrophoresis was performed in Tris-glycine buffer (pH 9.4) (Gabriel, 1971) or in sodium dodecyl sulfate, as suggested by Weber and Osborn (1969). Protein was visualized on polyacrylamide gels using Coomassie Brilliant Blue stain (Smith, 1968) and phosphatase activity was determined using α-naphthyl phosphate and Fast Red TR (Gabriel, 1971).

Metal Analysis. Two methods, spark emission spectrography and atomic absorption spectrometry, were employed for zinc and magnesium analysis. Primary working standards for metal analysis were prepared by dissolving weighed quantities of spectroscopically pure zinc rod and magnesium metal (Johnson, Matthey and Co., Ltd.) in metal-free HCl. To determine accuracy of the methods a zinc spelter, NBS sample 109; a copper-zinc-nickel alloy, NBS sample 157-A; a magnesium base alloy, NBS sample 171; and dolomite, NBS sample 88, all of certified metal content, were dissolved in metal-free HCl in the same manner as the aforementioned standards. All phosphatase samples were dialyzed for 4 days against multiple changes of a 100-fold excess of metal-free 0.01 *M* Tris-Cl (pH 8.0) to remove unbound metals.

For emission spectrography, 5–15 mg of protein was ashed in an electric muffle furnace at 450° for 24 hr (Thiers, 1957). The ash was dissolved in 6 *N* HCl containing 7 μg/ml of vanadium as an internal standard. Intensity ratio curves were constructed for each spectrographic plate by sparking, in triplicate, four standard concentrations of zinc, magnesium, and 15 other elements along with duplicate phosphatase samples (Vallee, 1955). For zinc concentrations between 1 and 30 μg/ml, the coefficient of variation for this method varies from 7 to 10% (Thiers and Vallee, 1957) and the accuracy is 100 ± 7%.

Atomic absorption spectrometry for zinc was performed with equipment previously described (Fuwa and Vallee, 1963). The coefficient of variation for this method is below 3% and the accuracy is 100 ± 5% (Fuwa et al., 1964). Phosphatase samples were ashed, then dissolved in 0.1 *N* HCl, and diluted with metal-free water to a zinc concentration of between 0.01 and 0.1 g/ml. For comparative purposes samples were analyzed prior to ashing to determine the effect of the organic matrix on zinc determination (Table I). In every case the zinc content of an ashed sample was found to be higher than the identical nonashed sample. Furthermore, the average zinc content of ashed samples, 2900 ± 180 μg/g or 4.0 ± 0.3 g-atoms/89,000, was 12% higher than that of the same nonashed samples, 2600 ± 190 μg/g or 3.6 ± 0.3 g-atoms/89,000.

Table I: Zinc Content of Alkaline Phosphatase as Determined by Atomic Absorption Analysis of Ashed and Nonashed Samples.

Pretreatment	Number of Samples[a]	Zinc	
		μg/g	g-atom/89,000 mol wt
Ashed	14	2900 ± 180	4.0 ± 0.3
Nonashed	14	2600 ± 190	3.6 ± 0.3

[a] Preparation numbers 13–26.

Atomic absorption spectrometry for magnesium was performed as described by Wacker et al. (1965). Samples were initially diluted with metal-free water to a magnesium concentration ranging from 0.05 to 0.5 μg/ml and finally 1:1 with 8-hydroxyquinoline (G. Fredrick Smith Chemical Co.) in 1 *N* HCl to obviate any interferences. The coefficient of variation for standards with this method is below 6% and the accuracy is 100 ± 1% (Iida et al., 1967). The metal content of phosphatase is expressed both in μg per g of protein and in g-atom of metal per molecular weight of 89,000 (Simpson et al., 1968) to facilitate comparsions with the results of these and other studies.

Preparation of Reagents. Analytical grade chemicals were used throughout, and all solutions were prepared with deionized and distilled water. All buffers were extracted with 0.001% diphenyldithiocarbazone in carbon tetrachloride until free of transition metals. In order to remove magnesium, all solutions extracted in this manner were treated further with Chelex 100. Glassware and polyethylene containers were rendered metal-free by soaking overnight in a 1:1 mixture of concentrated nitric and sulfuric acids, followed by copious rinsing with metal-free water (Thiers, 1957).

Results

Metal Content of Alkaline Phosphatase, 1962–1968. The earliest preparations of alkaline phosphatase in this laboratory (Plocke et al., 1962), obtained from whole cell extracts, contained zinc and magnesium as the major metal constituents, as measured by emission spectrography (Table II, no. 1–5). The zinc content ranged from 1100 and 2300 μg/g of protein, averaging 1700 μg/g or 2.1 g-atoms of zinc per molecular weight of 80,000 as determined by Garen and Levinthal (1960) or 2.3 g-atoms/89,000 as determined later by Simpson et al. (1968). The concentration of magnesium ranged from 190 to 300 μg/g of protein, averaging 240 μg/g or 0.8 g-atom/80,000 or 0.9 g-atom/89,000. However, the sample containing the highest amount of zinc, 3.1 g-atoms/89,000, also exhibited the highest activity, 41 units (Table II, 2). Several preparations contained variable but stoichiometric quantities of iron and calcium.

The purification scheme of Neu and Heppel (1965) releases alkaline phosphatase from the periplasmic space of *E. coli* using osmotic shock after treatment with EDTA and sucrose at pH 7.5. This procedure was employed to obtain preparations 6–12, Table I (Simpson et al. 1968). Preparations 6 and 7, obtained by crystallization from $(NH_4)_2SO_4$ and $MgCl_2$, had activities of 36 and 40 units. They contained 2000 and 2200 μg of zinc/g or 2.8 and 3.0 g-atoms/89,000 in addition to 550 and 760 μg of magnesium/g or 2.0 and 2.8 g-atoms/89,000. Preparations 8–12, Table II,

Table II: Metal Content of Alkaline Phosphatase Measured by Emission Spectrography (1962–1968).

Sample	Specific Activity (units)	Zinc μg/g	Zinc g-atom/80,000	Zinc g-atom/89,000	Magnesium μg/g	Magnesium g-atom/80,000	Magnesium g-atom/89,000
1[a]	24	1400	1.8	1.9	240	0.8	0.9
2	41	2300	2.8	3.1	190	0.6	0.7
3	24	1100	1.4	1.5	210	0.7	0.8
4	35	2000	2.4	2.7	300	1.0	1.1
5	39	1600	2.0	2.2	240	0.8	0.9
6[b]	36	2200		3.0	760		2.8
7	40	2000		2.8	550		2.0
8[c]	39	2500		3.5	430		1.6
9	43	3100		4.2	<10		<0.04
10	40	2800		3.9	560		2.0
11	39	2600		3.7	480		1.8
12	42	2700		3.8	520		1.9

[a] Preparations 1–5 are described by Plocke et al. (1962). In addition to zinc and magnesium, they contained 0.1–1.0 g-atom of iron and 0.3–1.2 g-atoms of calcium per 89,000. [b] Preparations 6–7 were crystallized from $(NH_4)_2SO_4$ (Simpson et al., 1968). In addition to zinc and magnesium, they contained 0.9 g-atom of iron and 0.2–0.6 g-atom of calcium per 89,000. [c] Preparations 8–12 are described by Simpson et al. (1968). In addition to zinc and magnesium they contained 0.6–1.0 g-atom of iron and <0.1–0.8 g-atom of calcium per 89,000.

were purified exclusively by column chromatography and had a specific activity of 39–43 units. The zinc content of these preparations was significantly higher than both the early preparations (Table II, no. 1–5), and the crystalline enzyme (Table II, 6 and 7). It ranged from 2500 to 3100 μg/g as determined by emission spectrography, with an average of 2700 μg/g or 3.8 g-atoms/89,000. Four of five preparations contained between 430 and 560 μg of magnesium/g or 1.6 to 2.0 g-atoms/89,000. Iron and calcium were the only other metals found in stoichiometric quantities.

Metal Content of Alkaline Phosphatase, Recent Preparations. The zinc content of phosphatase samples prepared from *E. coli* C-90 at pH 7.2 and 7.5 more recently, but in a manner identical with that reported by Simpson et al. (1968), ranged from 2600 to 3200 μg/g with an average of 2800 μg/g or 3.8 g-atoms/89,000, as determined by atomic absorption spectrometry of ashed samples (Table III, no. 13–16). The magnesium content ranged from 300 to 470 μg/g with an average of 350 μg/g or 1.3 g-atoms/89,000. Only zinc and magnesium were detected by emission spectrographic analysis, in quantities very similar to that found by atomic absorption analysis. For zinc the content ranged from 2400 to 3200 μg/g with an average of 2700 μg/g or 3.7 g-atoms/89,000, while for magnesium it ranged from 290 to 440 μg/g with an average of 360 μg/g or 1.3 g-atoms/89,000. The specific activity of preparations 13–16, Table III, was consistently high, 36–48 units, and no protein inhomogeneity could be detected by polyacrylamide gel electrophoresis.

Phosphatase preparations 17–20, Table III, were prepared by substituting Chelex 100 for EDTA to release the enzyme from the periplasmic space of *E. coli* C-90. The activity of these samples was between 36 and 45 units, values quite similar to those of enzyme released with EDTA (Table III, no. 13–16). By atomic absorption analysis of ashed samples, the zinc content ranged from 2700 to 3100 μg/g with an average of 3000 μg/g or 4.0 g-atoms/89,000 and by emission spectrography, it ranged from 2400 to 3400 μg/g with an average of 2800 μg/g or 3.9 g-atoms/89,000. By atomic absorption spectroscopy, the magnesium content ranged from 250 to 440 μg/g with an average of 350 μg/g or 1.3 g-atoms/89,000 and by emission spectrography, it ranged from 200 to 330 μg/g with an average of 270 μg/g or 1.0 g-atom/89,000. Again, zinc and magnesium were the only metals found in stoichiometric amounts. There is excellent agreement between the metal content of enzyme released by either EDTA or Chelex, whether measured by atomic absorption or emission spectrographic analysis.

Table III: Metal Content of Alkaline Phosphatase Isolated from *E. coli* C-90 and Measured by Emission Spectrography and Atomic Absorption Spectrometry.

Sample[a]	Specific Activity (units)	Zinc[b] Atomic Absorption μg/g	Zinc[b] Atomic Absorption g-atom/89,000	Zinc[b] Emission Spectroscopy μg/g	Zinc[b] Emission Spectroscopy g-atom/89,000	Magnesium[b] Atomic Absorption μg/g	Magnesium[b] Atomic Absorption g-atom/89,000	Magnesium[b] Emission Spectroscopy μg/g	Magnesium[b] Emission Spectroscopy g-atom/89,000
13	48	3200	4.4	2700	3.7	330	1.2	440	1.6
		3000	4.1	2600	3.5	320	1.2	290	1.1
14	46	3000	4.1	3200	4.4	310	1.1	380	1.4
		2900	3.9	2600	3.5	300	1.1	320	1.2
15	38	2700	3.7	2900	3.9	470	1.7	380	1.4
		2600	3.5	2600	3.5	470	1.7	330	1.2
16	36	2600	3.5	2700	3.7	310	1.1	350	1.3
		2600	3.5	2400	3.3	310	1.1		
17	45	2800	3.8	3000	4.1	280	1.0	240	0.9
		2700	3.7	2400	3.3	250	0.9	230	0.8
18	44	3100	4.2	2700	3.7	440	1.6	330	1.2
		3000	4.1	2400	3.3	440	1.6	300	1.1
19	38	3100	4.2	3400	4.6	350	1.3	210	0.8
		3100	4.2	3200	4.4	350	1.3	200	0.7
20	36	2900	3.9	2800	3.8	370	1.4	330	1.2
		2900	3.9	2700	3.7	340	1.2		
Av	41	2900	3.9	2800	3.8	350	1.3	310	1.2
SD	±5	±200	±0.3	±300	±0.4	±70	±0.3	±70	±0.3

[a] Samples 13–16 were prepared from periplasmic extracts using osmotic shock after treatment with EDTA and sucrose; samples 17–20 with Chelex 100 and sucrose. [b] Metal analysis was performed on samples ashed in an electric muffle furnace.

Table IV: Metal Content of Alkaline Phosphatase from *E. coli* Strains Measured by Atomic Absorption Spectrometry.

Sample	Strain	Specific Activity (units)	Zinc		Magnesium	
			μg/g	g-atom/ 89,000	μg/g	g-atom/ 89,000
21	K-10	41	3000	4.1	310	1.1
			2900	3.9	310	1.1
22	K-10	41	3200	4.4	320	1.2
			3100	4.2	310	1.1
23	C_4F_1	44	3000	4.1	410	1.5
			2800	3.8	380	1.4
24	C_4F_1	40	3100	4.2	450	1.6
			2800	3.8	420	1.5
25	CW-3747	40	3000	4.1	380	1.4
			2800	3.8	340	1.2
26	CW-3747	42	3000	4.1	400	1.5
			2800	3.8	380	1.4
Av		41	3000	4.0	370	1.3
SD		±2	±140	±0.2	±50	±0.2

[a] Metal analysis was performed on samples ashed in an electric muffle furnace.

Metal Content of Alkaline Phosphatase from Different E. coli Strains. Alkaline phosphatase was prepared from three different *E. coli* strains commonly employed for the isolation of this enzyme: K-10, an Hfr prototroph of *E. coli* K-12 in which phosphatase synthesis is repressed by inorganic phosphate; C_4F_1, a constitutive mutant carrying the phosphatase structural gene of K-10; and CW-3747, another constitutive mutant derived from K-12 and containing an episomal copy of the phosphatase structural gene, hence, yielding two to three times the amount of enzyme per gram of cells. The specific activity of all samples obtained from these three strains in duplicate preparations was between 40 and 44 units (Table IV, no. 21-26).

The zinc content ranged from 2800 to 3200 μg/g with an average of 3000 μg/g or 4.0 g-atoms/89,000 and the magnesium content ranged from 310 to 450 μg/g with an average of 370 μg/g or 1.3 g-atoms/89,000. These preparations do not differ significantly in zinc or magnesium content, or activity from those prepared from strain C-90 (Table III, 13-20).

Metal Content of Alkaline Phosphatase Isozymes. Since *E. coli* alkaline phosphatase is composed of multiple molecular forms, a periplasmic extract of *E. coli* C-90 obtained by EDTA-sucrose treatment at pH 7.5 (Neu and Heppel, 1965) was chromatographed on DEAE-cellulose at pH 7.2 (Figure 1) in accord with the procedure and data of Lazdunski and Lazdunski (1967) and Simpson et al. (1968). Isozyme 1, as isolated initially, had a specific activity of 9 units; it was rechromatographed with a resultant increase of the specific activity to 38 units. The zinc content of isozymes 1, 2, and 3, consecutively, was 2900, 2600, and 2200 μg/g of protein or 3.9, 3.5, and 3.0 g-atoms/89,000, and the magnesium content was 470, 470, and 380 μg/g or 1.7, 1.7, and 1.4 g-atoms/89,000 as determined by atomic absorption analysis while the activity was 38, 38, and 35 units (Table V).

Table V: Metal Content of Alkaline Phosphatase Isozymes.[a]

Sample	Specific Activity (units)	Zinc		Magnesium	
		μg/g	g-atom/ 89,000	μg/g	g-atom/ 89,000
Isozyme 1	9	1000	1.4	130	0.5
Isozyme 1 (purified)	38	2900	3.9	470	1.7
Isozyme 2	38	2600	3.5	470	1.7
Isozyme 3	35	2200	3.0	380	1.4

[a] Fractions containing the three separated isozymes (Figure 1) were concentrated and dialyzed against metal-free 0.01 *M* Tris-Cl (pH 8.0). The zinc and magnesium content of ashed samples was determined for each isozyme using atomic absorption spectrometry.

Discussion

The earliest metal analyses of alkaline phosphatase from *E. coli* (Plocke et al., 1962) indicated that preparations of enzyme then available contained an average of 2.1 g-atoms of zinc/80,000, as determined by emission analysis (Table II, no. 1-5). Since then, the zinc content of native phosphatase prepared and analyzed by a variety of procedures has been reported to range from 2.2 to 3.4, averaging 2.7 g-atoms (Csopak and Szajn, 1973) and from 2.5 to 3.5 g-atoms (Harris and Coleman, 1968), both based on a molecular weight of 80,000. Furthermore, Reynolds and Schlesinger (1968) reported an average zinc content of 3, ranging from 2 to 6 g-atoms and Lazdunski et al. (1969) found 4 g-atoms both per 86,000. Simpson et al. (1968) found an average zinc content of 3.7, ranging from 3.5 to 4.2 g-atoms/89,000. The first analyses of alkaline phosphatase (Plocke et al., 1962) also indicated the presence of 0.8 g-atom of magnesium/80,000 in addition to zinc (Table II, no. 1-5). Simpson et al. (1968) (Table II, no. 8-12) subsequently confirmed the presence of stoichiometric quantities of magnesium in phosphatase. Importantly, Plocke and Vallee (1962) postulated that zinc and magnesium affect enzymatic activity cooperatively. Nevertheless, this effect of magnesium on activity, metal content, or other chemical and physical properties of this enzyme generally has been disregarded. These circumstances, as well as the wide variation of the zinc content of phosphatase, reported to range from 2 to 6 g-atoms/mol, and of the molecular weight, ranging from 80,000 to 89,000, occasioned a detailed review of the data obtained in this laboratory and elsewhere, to examine all factors pertinent to the preparation and analysis of the metal content of this enzyme.

Three general considerations are germane to the evaluation of the metal content of biological materials: (1) features intrinsic to the analytical methodology as expressed by its repeatability, precision, and accuracy; (2) calculation and interpretation of results in terms of mass or molar ratios; and (3) selection of a representative biological sample. It would seem appropriate to examine the problem in these terms.

The evaluation of analytical methods is accomplished most readily both by employing absolute standards of accuracy, provided by the National Bureau of Standards, and by statistical approaches. The inherent characteristics of emission spectrography have proven valuable for the study of metalloenzymes, since concentrations of many elements may be determined simultaneously (Vallee, 1955). Over the range from 1 to 30 μg of zinc/ml the porous cup procedure employed in these studies has a coefficient of variation of 7-10% (Thiers and Vallee, 1957). Photography and densitometry account for this deviation, but during continuous use of the method the precision has remained constant. However, atomic absorption spectrometry has become the

method of choice for the determination of zinc or magnesium. It is at least 10 to 100 times more sensitive than emission spectrography and allows the use of correspondingly lesser quantities of material, an important consideration in the metal analysis of enzymes.

For certain proteins, atomic absorption measurements can be performed directly after dilution of the protein solutions and without prior destruction of organic material. However, for alkaline phosphatase, the zinc content of ashed samples is consistently 12% higher than that of identical samples analyzed prior to ashing (Table I). Hence, for interpretation of data it is essential to know whether preparations reported to contain from 2 to 6 g-atoms of zinc as measured by atomic absorption spectrometry (Harris and Coleman, 1968; Reynolds and Schlesinger, 1968; Lazdunski et al., 1969; Csopak and Szajn, 1973) were ashed prior to analysis or analyzed directly.

For the analysis of magnesium by atomic absorption spectrometry, the inclusion of 8-hydroxyquinoline as a spectral buffer obviates interferences by the organic matrix as verified by analysis of both ashed and nonashed samples. The accuracy of zinc analysis, 100 ± 3% (Fuwa et al., 1964), and that of magnesium, 100 ± 1% (Iida et al., 1967), is remarkable considering that absolute metal concentrations as low as 0.01 μg/ml can be measured.

The average zinc content of the recent alkaline phosphatase preparations (Table III, 13–20) was 2800 ± 300 μg/g or 3.8 ± 0.4 g-atoms/89,000, while the average magnesium content was 310 ± 70 μg/g or 1.2 ± 0.3 g-atoms/89,000, as determined by emission spectrography, regardless of the specific activity or mode of preparation. The difference between the zinc content of these preparations and that reported by Simpson et al. (1968) (Table II, no. 8–12) as compared to the initial preparations of Plocke et al. (1962) (Table II, no. 1–5) cannot be attributed to the analytical methodology but would appear to be intrinsic to the samples analyzed. The average zinc content for atomic absorption analysis of ashed samples (Tables III and IV, no. 13–26) was 2900 ± 180 μg/g or 4.0 ± 0.3 g-atoms/89,000, while that for magnesium was 360 ± 60 μg/g or 1.3 ± 0.2 g-atoms/89,000, regardless of the bacterial strain from which the enzyme was isolated, its specific activity, or mode of preparation. While both the smaller difference between duplicates and lower standard deviation of atomic absorption analysis demonstrate that the method is more reproducible than emission analysis, the two analytical methods are in good agreement for both zinc and magnesium. Alkaline phosphatase of *E. coli* C-90 has also been examined by microwave induced emission spectroscopy (Auld et al., 1974; Kawaguchi and Vallee, 1975). The material contained 4.1 g-atoms of zinc and 1.4 g-atoms of magnesium. These values are entirely consistent with those reported in Tables III and IV, confirming our conclusion that the same values are intrinsic to the samples analyzed and, hence, not attributable to the analytical methods used.

The choice of suitable units for the description of the metal content of proteins is critical. Conventionally, the metal content of phosphatase has been expressed in units of g-atom per mole of protein and, hence, is dependent upon the value of the molecular weight utilized for such calculations. Molecular weight values for alkaline phosphatase have varied from 80,000 to 100,000 (Garen and Levinthal, 1960; Schlesinger and Barrett, 1965; Simpson et al., 1968; Applebury and Coleman, 1969; Trotman and Greenwood, 1971) resulting in a proportionate variation in the value of g-atom of metal per mole of enzyme. For example, while the average zinc content of preparations 1–5, Table II, was reported to be 2.1 g-atoms/mol, based upon initial molecular weight estimates of 80,000 as reported by Garen and Levinthal (1960), it increases to 2.3 g-atoms based on the molecular weight of 89,000, as later determined by Simpson et al. (1968). Csopak and Szajn (1973) concluded that alkaline phosphatase contains 2 g-atoms of zinc. Actually the data reported from 2.2 to 3.4 g-atoms of zinc based on a molecular weight of 80,000. When recalculated based on a molecular weight of 89,000 this increases to range from 2.5 to 3.8 g-atoms/mol. Similarly, Harris and Coleman (1968) report the zinc content to be 2.5–3.5 g-atoms/80,000 which becomes 2.8–3.9 g-atoms/89,000. These values are quite similar to those found for nonashed samples (Table I), i.e., 3.6 ± 0.3 g-atoms/89,000.

It should be noted, moreover, that the values of protein absorbance utilized for calculations of phosphatase concentration have varied from an $A_{278}(1\%)$ of 7.0 (Plocke et al., 1962) to 7.2 (Malamy and Horecker, 1964; Simpson et al., 1968; Harris and Coleman, 1968; Csopak et al., 1972b) to 7.7 (Lazdunski et al., 1969) and to an $A_{280}(1\%)$ of 7.7 (Rothman and Byrne, 1963; Schlesinger and Barrett, 1965). Obviously, the metal content, when based on an $A_{278}(1\%)$ of 7.7, would be 7% higher than that based on a value of 7.2.

The selection of a biological sample, while simple in principle, can also present significant, practical problems. Few, if any, protein preparations satisfy all available criteria of purity (Sober et al., 1965). Different preparations of the same enzyme, isolated by the same procedure and homogeneous in all known respects, have proven to be dissimilar when it became possible to examine new properties. Thus, the selection of a truly representative sample of a given protein can be beset with uncertainty.

The specific activities of the initial preparations of phosphatase (Table II, no. 1–5) analyzed for metal in this laboratory varied from 24 to 41 units (Plocke et al., 1962), perhaps reflecting an undetermined protein heterogeneity. The specific activity of more recent preparations, ranging from 36 to 48 units (Tables III and IV, no. 13–26), is consistently higher, in accord with electrophoretic and chromatographic analyses which demonstrate that these preparations are free of extraneous proteins. The recent preparations are not only more active, but also contain more zinc. Thus, sample number 13, Table II, has both the highest activity and the highest zinc content. However, the variation in specific activity of alkaline phosphatase samples does not correlate exclusively with zinc content but, in addition, is affected by other parameters. Indeed, Plocke and Vallee (1962) reported that the presence of magnesium increases the activity of the zinc enzyme. The past and present data strongly support the suggestion that magnesium modulates the activity of the zinc enzyme and hence, the specific activity likely reflects the cooperative effect of these two metals which has become the subject of detailed examination (Anderson et al., 1975).

Metal contamination or loss has also presented problems in the evaluation both of the composition and activity of metalloenzymes (Vallee and Wacker, 1970). High concentrations of zinc and/or magnesium when present in the course of purification by means of heat treatment (Garen and Levinthal, 1960; Plocke et al., 1962; Applebury and Coleman, 1968; Csopak and Szajn, 1973) or by means of $(NH_4)_2SO_4$ crystallization or precipitation (Malamy and Horecker, 1964; Applebury and Coleman, 1969) or in buff-

ers (Schlesinger and Olsen, 1970; Csopak et al., 1972b) might be expected to alter the intrinsic metal composition of the enzyme. Some of the preparations in Table II contain significant concentrations of metals other than zinc or magnesium. If these metals represent contaminants, they could displace either zinc, magnesium, or both from native phosphatase, thereby affecting its activity. Moreover, changes in pH during dialysis against metal-free water to remove contaminating metals from phosphatase (Reynolds and Schlesinger, 1968) might also inadvertently remove intrinsic metal. Samples recently prepared under conditions designed to minimize extraneous metal contaminants and to prevent loss of intrinsic metals (Tables III and IV, no. 13–26) solely contain zinc and magnesium in stoichiometric amounts and no other metals, as determined by emission spectrography.

The effect of various purification procedures upon the metal composition of alkaline phosphatase is well documented. Phosphatase prepared by crystallization from $(NH_4)_2SO_4$ and $MgCl_2$ (Table II, no. 6 and 7) contains less zinc, 2.8–3.0 g-atoms, and more magnesium, 2.0–2.8 g-atoms (vide supra), than that prepared without exposure to these agents (Tables II–IV, no. 8–26). It was suggested that such procedures could remove zinc by formation of a stable $Zn(NH_3)_4^{2+}$ complex (Simpson et al., 1968), a circumstance which could affect the interpretation of X-ray analysis of phosphatase crystallized from $(NH_4)_2SO_4$ (Knox and Wyckoff, 1973).

We have reported that EDTA, utilized routinely in the preparation of apoenzyme by some investigators, binds to the protein and is removed only by prolonged dialysis (Ulmer and Vallee, 1971), an observation subsequently confirmed by Csopak et al. (1972a). Csopak and Szajn (1973) also reported that EDTA, when utilized to release phosphatase from the periplasmic space, remains bound firmly to the enzyme throughout all stages of purification, thereby providing additional zinc binding sites. These, in turn, could account for the discrepancies in metal stoichiometry detailed here. However, the use of isolation procedures which never expose the enzyme to chelating agents results in preparations with an average zinc content of 4.0 ± 0.2 g-atoms/mol (Table III, no. 17–20), identical with enzyme released from the periplasmic space in the presence of EDTA and containing an average of 4.0 ± 0.3 g-atoms/mol (Tables III and IV, no. 13–16 and 21–26).[2]

In addition to the possibility that the reported discrepancies in the metal content of phosphatase result from variations in analytical methodology, expression of results or purification techniques, the possibility was examined that such variations are inherent to the *E. coli* strain utilized for enzyme production. Atomic absorption analysis of purified alkaline phosphatase isolated from three commonly utilized *E. coli* strains, K-10, C_4F_1, and CW-3747, indicates the presence of 4.0 ± 0.2 g-atoms of zinc and 1.3 ± 0.2 g-atoms of magnesium/mol in all of these (Table IV, no. 21–26).

Purified phosphatase was separated further into three forms by high resolution DEAE-cellulose chromatography (Figure 1). The similarity of metal content, ranging from 3.0 to 3.9 g-atoms of zinc and 1.4 to 1.7 g-atoms of magnesium/mol (Table V), indicates that any variation in metal content of a mixture of isozymes cannot result from inherent differences in the metal content of the individual forms.

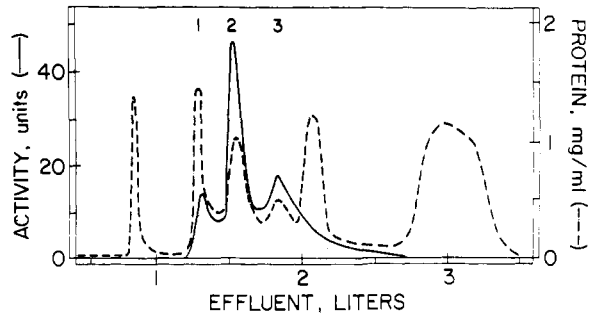

FIGURE 1: Separation of phosphatase isozymes on DEAE-cellulose. A periplasmic extract from 100 l. of *E. coli* C-90, grown and harvested as described in the Experimental Section, was prepared utilizing osmotic shock in EDTA and sucrose. The extract was absorbed onto 50 g of DEAE-cellulose, eluted with 0.1 *M* NaCl in 0.01 *M* imidazole-Cl (pH 7.2), and concentrated by ultrafiltration (Amicon PM-10 membrane). The protein, 1.9 g, was applied to a 2.5 × 40 cm column of DEAE-cellulose, washed with 0.25 l. of 0.02 *M* NaCl in buffer, and eluted with a 4-l. linear gradient of 0.02–0.1 *M* NaCl in buffer. Protein concentration (- - -) and enzymatic activity in μmole per min per ml (—) are plotted.

The methods available both for the isolation and physicochemical characterization of proteins necessarily place further constraints upon the accuracy with which any given property can be measured and the confidence with which it can be correlated meaningfully to others. This is exemplified by *E. coli* alkaline phosphatase, and the reported variations in the values for molecular weight, maximal specific activity, absorbance at 278 nm, as well as the stoichiometry of zinc and magnesium. The uncertainties concerning the molar stoichiometry of zinc in phosphatase over the past 13 years appear to be attributable largely to differences in analytical methodology, the molecular weight values utilized in calculating the molar metal content, and changes in purification techniques affecting the homogeneity and intrinsic metal content of the protein. The present data show that native *E. coli* alkaline phosphatase, when isolated and purified to homogeneity at pH 7.2 and 7.5 in a manner so as to avoid contamination or loss of metal, contains 4.0 ± 0.3 g-atoms of zinc and 1.3 ± 0.2 g-atoms of magnesium/phosphatase molecular weight 89,000 (Tables III and IV, no. 13–26).

Importantly, previous studies on the stoichiometry of metals in phosphatase have completely ignored the presence of magnesium. Equilibrium dialysis experiments evaluating the binding of metal to phosphatase demonstrate that the quantity of magnesium bound to apoenzyme depends critically both upon pH and zinc content (F. S. Kennedy, W. F. Bosron, R. A. Anderson, and B. L. Vallee, in preparation). In fact, phosphatase containing 4 g-atoms of zinc will bind 1.4 g-atoms of magnesium at pH 7.2, while at higher pH values even more magnesium is bound. Hence, the 1.3 g-atoms of magnesium found for the enzyme preparations reported here is entirely consistent with the pH dependence of magnesium binding to apoenzyme. The present data and others to be reported indicate that, maximally, alkaline phosphatase of *E. coli* contains a total of 6 g-atoms of metal, i.e., 4 zinc and 2 magnesium.

We have previously indicated the importance of defining

[2] The interaction of EDTA with alkaline phosphatase was examined throughout the course of purification following periplasmic release in the presence of 0.1 mCi of [^{14}C]EDTA in accord with the procedure of Csopak and Szajn (1973). Only 1% or 1.4 mol of [^{14}C]EDTA remained associated with the enzyme after batchwise purification on DEAE-cellulose and followed by concentration. Subsequent chromatography on DEAE-cellulose indicates that the [^{14}C]EDTA does not elute conjointly with the phosphatase activity but closely *follows* it as a symmetrical peak upon application of higher salt.

the role of magnesium in the function of alkaline phosphatase (Plocke and Vallee, 1962; Simpson et al., 1968). A detailed examination of the effect of magnesium on the zinc enzyme, in fact, resolves previous ambiguities concerning the quantity of zinc required for maximal activity and stability of alkaline phosphatase. Magnesium alone does not activate the apoenzyme, but the presence of stoichiometric quantities of magnesium regulates the mode of zinc binding and thereby increases enzymatic activities over those attained in the presence of zinc alone (F. S. Kennedy, W. F. Bosron, R. A. Anderson, and B. L. Vallee, in preparation). Similar results are obtained with enzymes in which cobalt is substituted for zinc (R. A. Anderson, F. S. Kennedy, and B. L. Vallee, in preparation). Furthermore, the presence and effects of magnesium bear importantly on the many spectral and kinetic investigations employing metal substitution with Co^{2+}, Cu^{2+}, Mn^{2+}, and other divalent metal atoms aiming at correlation of composition and structure of the enzyme with its function. The spectra of cobalt phosphatases, in fact, permit the visualization of the effects of magnesium on cobalt coordination geometry. In the presence of magnesium, the spectral characteristics of the first two g-atoms of cobalt predominantly reflect tetra- or pentacoordinate-like geometry, but in its absence, the cobalt atoms redistribute between tetrahedral- and octahedral-like sites as evidenced by absorption, magnetic circular dichroic, and electron paramagnetic resonance spectra. The present analytical considerations concerning the metal composition of alkaline phosphatase pertain directly to the enzyme's chemical and functional properties which have been discussed elsewhere (Anderson et al., 1975).

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