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Alkaline Phosphatase of *Escherichia coli*: A Zinc Metalloenzyme*

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Escherichia coli alkaline phosphatase contains firmly bound zinc in high concentration. The average value for five different preparations was 2.1 gram atoms per mole of enzyme of molecular weight 80,000. During the steps of purification the zinc concentration rises steadily to a high value in the final product, while the concentrations of all the other metals decrease to low values. Inhibition of enzymatic activity by a number of metal-binding agents follows the order expected on the basis of the known stability constants of their soluble complexes with zinc ions. The instantaneous inhibition by sodium cyanide is reversible both on dilution and on addition of metal ions. These data all support the conclusion that zinc is a firmly bound component of the enzyme molecule essential to enzymatic activity.

A number of investigations have shown that metal ions function in the catalytic action of mammalian alkaline phosphatases, but the evidence for this is indirect (Erdtmann, 1927; Cloetens, 1941; Sadasivan, 1952; Portmann, 1957; Kunitz, 1960). However, direct analytical verification by measurement of the metal content of such enzymes is recent: the alkaline phosphatases of swine kidney (Mathies, 1958), calf intestine (Engström, 1961), and human leukocytes (Trubowitz *et al.*, 1961) contain 0.15%, 0.2%, and 0.7%[1] zinc respectively; the enzymatic activity of each enzyme is related to its metal content.

The alkaline phosphatase of *E. coli* is inhibited by metal-binding agents (Garen and Levinthal, 1960). This finding led to the present investigation of the role of a metal and the demonstration of zinc as a functional component of the enzyme.

METHODS AND MATERIALS

Reagents.—The method of cleaning glassware and the preparation of metal-free water were as previously described (Thiers, 1957). Tris (Sigma 121) was dissolved in metal-free water and adjusted to *p*H 8.0 with metal-free HCl. DEAE-cellulose (Eastman Lot No. 1056) was washed extensively with HCl, NaOH, and metal-free water before equilibration with Tris buffer at the desired *p*H. *p*-Nitrophenyl phosphate (Sigma 104) was dissolved in metal-free, distilled water. Ethylenediaminetetraacetic acid (EDTA), 8-hydroxyquinoline, 1,10-phenanthroline, sodium cyanide, sodium sulfide, and sodium diethyldithiocarbamate were all analytical grade reagents dissolved in Tris buffer and adjusted to *p*H 8.0 before use.

Protein concentration was determined gravimetrically after precipitation with trichloroacetic acid (Hoch and Vallee, 1953) and by absorbancy at 278 mμ. The molar absorbancy index (a_m),

[1] Calculated from the reported ratio of 44.1 μg/mg N, assuming a 16% nitrogen content for the protein.

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based on a weight obtained after trichloroacetic acid precipitation, is 5.6×10^4, using the reported molecular weight of the enzyme of 80,000 (Garen and Levinthal, 1960).

Metal content of all fractions was determined by emission spectrography (Vallee and Hoch, 1955).

Enzymatic activity was assayed at 25° by following the release of *p*-nitrophenol from *p*-nitrophenyl phosphate through the change in absorbancy at 410 mμ (Garen and Levinthal, 1960) in a Beckman DU spectrophotometer equipped with a thermostatically controlled chamber. The enzyme was added last to give a concentration of about 1 μg per ml, in a cuvet containing a final volume of 3 ml of 10^{-3} M *p*-nitrophenyl phosphate in 1 M Tris buffer, *p*H 8.0. Readings

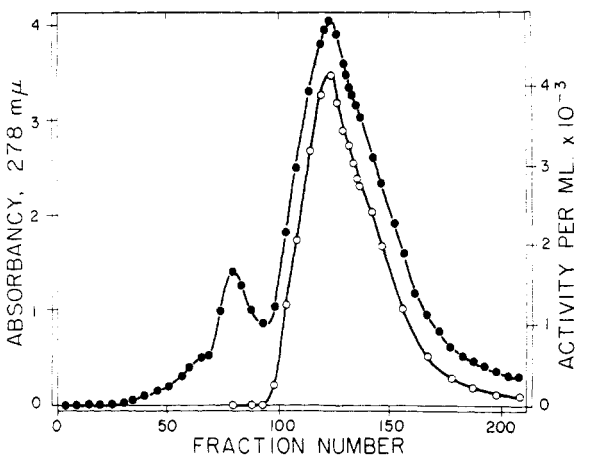

FIG. 1.—DEAE-cellulose column fractionation of *E. coli* alkaline phosphatase. A 4.5-cm column was packed to a height of 30 cm with DEAE-cellulose previously equilibrated with 0.01 M Tris, pH 7.4. After addition of the material containing the enzyme, 2 column volumes of buffer were washed through, followed by a gradient of 0-0.12 M NaCl in 0.01 M Tris, *p*H 7.4, fractions of 10 ml being collected as shown in the figure. Absorbancy at 278 mμ, ●; total enzymatic activity per ml, ○.

were taken every 10 seconds during the first minute of reaction. When the total change in absorbancy was less than 1.0, linear reaction rates were observed. Specific activity was expressed as change in absorbancy (V) per minute per mg of enzyme per ml of assay mixture volume. Using a measured molar absorbancy index for *p*-nitrophenol in 1 M Tris, *p*H 8.0, of 1.62×10^4 and a molecular weight for the enzyme of 80,000, a specific activity of 700 corresponds to a turnover number of 3200 moles of substrate hydrolyzed per mole of enzyme per minute.

Enzyme Purification.—Cells of *E. coli* K_{12}, either wild-type or constitutive for phosphatase (Garen and Levinthal, 1960; Torriani and Rothman, 1961) were employed. Preparations I and III were obtained from wild-type and constitutive strains respectively. These cultures were grown in an American Sterilizer Biogen unit with a lactate–inorganic salts medium. The harvested cells were extracted in 0.02 M tris-(hydroxymethyl)aminomethane buffer, *p*H 7.4, with a French pressure cell. Preparation II was supplied in partially purified form by the Worthington Biochemical Corporation and further purified by DEAE-cellulose column chromatography. Preparations IV and V were obtained from cells of the constitutive mutant grown by the Grain Processing Corporation, Muscatine, Iowa, using a tryptone-yeast extract medium. An acetone powder was prepared in our laboratory from the frozen cells.

In all instances except preparation II, the method of purification was similar to that published previously (Garen and Levinthal, 1960). A cell extract was obtained with the aid of the French pressure cell or by extraction of an acetone powder for 1 hour at room temperature in 0.005 M Tris buffer, *p*H 7.4, followed in either case by centrifugation in the no. 21 rotor of the Spinco Model L ultracentrifuge at 17,000 rpm for 1 hour. Before centrifugation 1 mg each of deoxyribonuclease and ribonuclease were added for each kilogram of the original starting material. After centrifugation the supernatant was dialyzed against 0.01 M Tris, *p*H 7.4, adsorbed on a DEAE-cellulose column at this *p*H, washed extensively on the column with Tris buffer, and eluted with 0.12 M NaCl in 0.01 M Tris, *p*H 7.4. $MgSO_4$ was added to the enzymatically active portions of the eluate to result in a concentration of 10^{-2} M, and after heating to 80° for 7 minutes, the resulting flocculent precipitate was removed by centrifugation or filtration and the clear supernatant dialyzed and again adsorbed on a DEAE-cellulose column. Elution was performed using a gradient from 0 to 0.12 M NaCl in 0.01 M Tris, *p*H 7.4. The fractions of highest activity were pooled for analysis and fractions of lower activity pooled for rechromatography. Figure 1 shows the results of the gradient elution step for preparation V. Samples were removed at each stage for spectrographic analysis and for measurement of protein concentration and enzymatic activity. This method of purification has been shown to yield preparations which are homogeneous according to several different criteria; measurements of molecular weight by two independent methods were in close agreement (Garen and Levinthal, 1960; Hall, 1960).

RESULTS

Metal Analyses.—Table I presents spectrographic analyses of five different preparations of *E. coli* alkaline phosphatase. Analyses were performed on materials with the highest enzymatic activity after DEAE-cellulose column chromatography and extensive dialysis against 0.01 M Tris buffer, *p*H 7.4. Zinc, the major metallic constituent, varies in concentration from 1100 to 2300 μg per gram of protein. The molar ratios of zinc to protein range from 1.4 to 2.8, averaging 2.1, based on a molecular weight of 80,000 (Garen

TABLE I

METAL CONTENT AND ACTIVITY OF PURIFIED PREPARATIONS OF *E. coli* ALKALINE PHOSPHATASE
Metal content is given in micrograms per gram of protein unless indicated otherwise

Prepn.	Specific Activity[a]	Zn (g atoms per mole protein)	Zn	Fe	Mg	Ca	Al	Cu	Ni	Mn	Ba	Sr	Cr
I	403	1.8	1400	650	240	320	130	[b]	59	34	23	4	—[c]
II	695	2.8	2300	51	190	520	97	[b]	30	15	10	6	3
III	405	1.4	1100	370	210	210	94	[b]	6	17	2	2	21
IV	590	2.4	2000	150	300	170	20	38	42	99	6	—[c]	—[c]
V	670	2.0	1600	88	240	150	19	130	—[c]	83	3	—[c]	13

[a] Specific Activity = ΔA_{410} per min per mg protein per ml. [b] Not determined. [c] Not detected; Be, B, Cd, Co, Li, Mo, Ag, Pb, and Sn were not detected in any sample.

and Levinthal, 1960). The concentration of magnesium, the only other element consistently present in significant amounts, ranges from 190 to 300 μg per gram of protein, averaging 0.78 gram atoms per mole of protein. The concentrations of calcium, iron, manganese, copper, and aluminum vary widely from one preparation to another; the iron content, for example, varies from 51 μg per gram of protein in preparation II to 650 in preparation I.

The relationship of zinc to enzymatic activity is shown in Table II. The metal content and enzymatic activity of consecutive fractions obtained during a preparation of alkaline phosphatase from an acetone powder suspension of *E. coli* (preparation V) are shown. Specific activity rises progressively from 11.4 units per mg protein per ml in the cell extract to 670 units per mg protein per ml in the final product. The zinc content rises from 0.25 gram atoms in the cell suspension to 2.0 gram atoms per molecular weight in the final product. The ratio of activity to zinc also rises progressively from 0.023 to 0.42 units per mμg Zn per ml. In contrast, the individual concentrations of all the other elements, as well as their sum, *decrease* with purification (see Table II). In only a few instances do increases in concentration occur between purification steps. Magnesium increases in fraction 4, corresponding to the preparative addition of magnesium sulfate at this stage. Both copper and lead, which increase concurrently in the same fraction, are reduced sharply in the next stage of purification.

At the bottom of Table II are shown fractions, 5a and 5b, which have low enzymatic activity and are eluted from the DEAE-cellulose column prior to, and following, the emergence of highly active material. Both fractions contain relatively little zinc, while other metals, particularly iron and calcium, are concentrated in comparison with the relatively small amounts of these metals in the material of the final product, fraction 5c.

Inhibition by Metal-Binding Agents.—The role of zinc in the enzymatic action of *E. coli* alkaline phosphatase was investigated by the use of agents known to bind metal ions in solution.

TABLE II

METAL ANALYSES OF FRACTIONS DURING PURIFICATION OF *E. coli* ALKALINE PHOSPHATASE
Metal content of all metals other than zinc in micrograms per gram of protein only

	Fraction	Specific Activity (*V*/mg protein/ml)	Zinc-Protein Ratio μg/g protein	Zinc-Protein Ratio g atoms/mole protein	Activity-Zinc Ratio (*V*/mμg Zn/ml)	Mg	Fe	Ca	Al	Cu	Ni	Mn	Be	Si	Cr
1	Acetone powder suspension	[a]	200	0.25	[a]	880	2300	1700	43	210	110	170	12	6	30
2	Dialyzed supernatant of cell extract	11.4	490	0.60	0.023	1270	500	1000	30	90	—[b]	140	22	5	24
3	Pooled active fractions, 1st DEAE-cellulose column	114	650	0.81	0.17	140	340	280	29	62	—[b]	95	6	—[b]	12
4	Supernatant from heat-denatured material[c]	272	1100	1.3	0.25	1300	270	210	45	1200	—[b]	89	48	3	—[b]
5c	Highest activity fractions	670	1600	2.0	0.42	240	88	150	19	130	—[b]	83	83	—[b]	13
5a	2nd DEAE-cellulose column; material eluted before enzyme	0.2	220	0.27	0.001	140	720	2300	220	[a]	—[b]	—[b]	9	—[b]	99
5b	2nd DEAE-cellulose column; material eluted after main peak	60	560	0.69	0.11	240	790	2000	170	[a]	320	48	100	16	180

[a] Not determined. [b] Not detected; Be, B, Cd, Co, Li, Mo, Ag, Sn, Pb were not detected in any sample. [c] This sample also contained 405 μg Pb/g protein.

Incubation of the enzyme with ethylenediaminetetraacetic acid, 8-hydroxyquinoline, 1,10-phenanthroline, sodium sulfide, sodium cyanide, and sodium diethyldithiocarbamate for one hour results in inhibition of enzymatic activity. Table III shows the concentration of each of these

TABLE III

EFFECT OF METAL-BINDING AGENTS ON *E. coli* ALKALINE PHOSPHATASE ACTIVITY

The enzyme (1.6×10^{-8} M) was incubated with inhibitor for 1 hour in 1 M Tris buffer, *p*H 8.0, 25°. For assay, 0.1 ml *p*-nitrophenyl phosphate (0.03 M) was added to 2.9 ml incubation mixture and the reaction velocity was determined during the first minute of reaction by following the absorbancy at 410 mμ.

Agent	Concentration for 50% Inhibition (M)
Ethylenediaminetetraacetic acid	6×10^{-7}
8-Hydroxyquinoline	3×10^{-5}
1,10-Phenanthroline	4×10^{-5}
Sodium sulfide	6×10^{-5}
Sodium cyanide	6×10^{-4}
Sodium diethyldithiocarbamate	3×10^{-3}

agents required to produce 50% inhibition. The *effective* concentrations of three of these inhibitors, 8-hydroxyquinoline, 1,10-phenanthroline, and sodium cyanide, were calculated from the reported values for their acid dissociation constants (Bjerrum *et al.*, 1957 and 1958). The observed inhibitions are plotted as a function of the log of

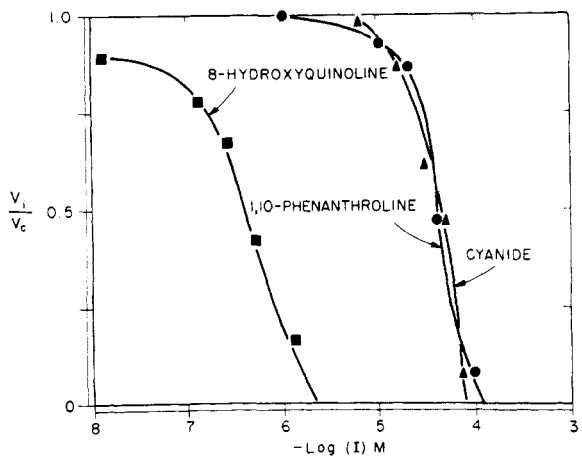

FIG. 2.—Inhibition of *E. coli* alkaline phosphatase as a function of the effective concentration of metal-binding agents. Incubation and assay were performed as described in Table III. The effective concentrations of inhibitor were determined by calculating the amount of free (unprotonated) inhibitor present at *p*H 8.0, using the values for the acid dissociation constants for the respective inhibitors. No correction was required for 1,10-phenanthroline. 8-Hydroxyquinoline, ■; 1-10-phenanthroline, ●; sodium cyanide, ▲.

the *effective* inhibitor concentration in Figure 2. Finally, in Table IV, values of K_I (the concentration required to produce 50% inhibition, from Figure 2) are compared with values of K_{Zn}, the stability constants for the ionic zinc complexes of these agents. The order for the series of $-\log K_I$ and $\log K_{Zn}$ is identical. Not included in this comparison are sulfide and diethyldithiocarbamate, since their complexes are insoluble, and ethylenediaminetetraacetic acid, because of uncertainty in the calculation of the effective concentration of the complexing species.

Although certain of the above agents, *e.g.* 1,10-phenanthroline and diethyldithiocarbamate, require preincubation for inhibition of enzymatic activity, sodium cyanide and sodium sulfide effect complete inhibition *instantaneously*. "Instantaneous" refers here to measurements performed as rapidly as activity can be measured on addition of enzyme to inhibitor and substrate, *i.e.* usually within 10 seconds. Figure 3 shows the instantaneous inhibition by cyanide as a function of the log of the concentration of cyanide. On dilution of the assay mixture by addition of substrate and buffer, this inhibition is reversed. For instance, in an assay mixture containing 0.015 M NaCN the enzyme exhibits 32% of the activity

TABLE IV

COMPARISON OF THE ORDER OF STABILITY CONSTANTS OF METAL-BINDING AGENTS AND THEIR ORDER OF EFFECTIVENESS AS INHIBITORS OF *E. coli* ALKALINE PHOSPHATASE

Agent	$-\log K_I$[a]	$\log K_{Zn}$[b]
8-Hydroxyquinoline	6.4	20.8[c]
1,10-Phenanthroline	4.4	17.0
Sodium cyanide	4.4	16.9

[a] K_I = Effective concentration of inhibitor required to produce 50% inhibition as determined from Figure 2. [b] $K_{Zn} = \frac{(Zn \cdot L_n)}{(Zn^{++})(L)^n}$; values from Bjerrum *et al.*, 1957, 1958. [c] A number of values of K have been reported, ranging from 17.6 to 20.8 (Bjerrum *et al.*, 1957, 1958).

of a control containing no inhibitor. When this mixture is diluted fivefold to an inhibitor concentration of 0.003 M, relative activity increases to 67%. Addition of enzyme directly to an assay mixture containing 0.003 M NaCN results in activity which is 76% of a control, indicating that reversal is nearly complete.

The instantaneous inhibition with cyanide is also reversed when zinc ions are added (Fig. 4). Reversal is complete at a ratio of zinc to cyanide of 1:2. Table V shows the results of adding Cu^{++}, Cd^{++}, and Mg^{++} ions in a ratio of metal ions to cyanide of 1:4, a ratio which with zinc ions effects a 92% reversal of the inhibition. Although in the absence of cyanide, addition of Cu^{++} or Cd^{++} ions alone results in a slight inhibition of activity, both of these metal ions

reverse the cyanide inhibition. Under these conditions magnesium ions, which do not form complexes with cyanide, neither affect the activity of the uninhibited enzyme nor reverse the inhibition with cyanide.

Discussion

The most highly purified preparations of *E. coli* alkaline phosphatase contained at least 2.0 gram atoms of zinc per mole of protein; the average value for five different purified preparations was 2.1 ± 0.5 gram atoms per mole of enzyme. Preparation no. II contained 2.8 gram atoms of zinc, while no. V, exhibiting comparable activity, contained only 2 gram atoms of zinc, suggesting that no. II may have contained extraneous zinc. In preparations I and III, respectively, where less than 2 gram atoms of zinc were found, the specific activity was correspondingly lower. These results suggest a value of 2 gram atoms of zinc per mole of enzyme; however, this tentative conclusion requires further confirmation.

The concentrations of all metals other than zinc decreased during purification; zinc, on the other hand, undergoes an approximately eightfold aggregation during purification of the enzyme. In preparation V the copper content rises suddenly in one step (Table II); this probably represents contamination, since the same sample also contained appreciable amounts of lead, an element not even detected in any other sample analyzed. Apart from magnesium, the metals other than zinc found in the purified preparations are also almost certainly contaminants, since the amounts of each are extremely variable from one preparation to another and generally correspond to far less than 1 gram atom of metal per mole of protein. Although magnesium was found in most preparations in amounts approaching stoichiometric significance, positive correlation between magnesium content and activity could not be discerned. The problem of assigning functional significance to the presence of magnesium is complicated further since 10^{-2} M $MgSO_4$ is added in the course of purification to stabilize the enzyme during the heat denaturation step; its presence in the final product may be attributed directly to this preparative step. It may be significant that the preparation exhibiting the greatest specific activity and highest zinc content, preparation II, had the lowest magnesium content, corresponding to only 0.62 gram atoms per mole enzyme. The data thus suggest that mag-

Table V

Reversal of the Instantaneous Inhibition of *E. coli* Alkaline Phosphatase with Cyanide by Addition of Metal Ions

NaCN Conc. (M)	Metal Conc. (M)	V_i/V_c	% Reversal
0	0	1.00	—
10^{-2}	0	0.45	—
0	2.5×10^{-3} Zn^{++}	1.00	—
10^{-2}	2.5×10^{-3} Zn^{++}	0.96	92
0	2.5×10^{-3} Cu^{++}	0.93	—
10^{-2}	2.5×10^{-3} Cu^{++}	0.84	71
0	2.5×10^{-3} Cd^{++}	0.92	—
10^{-2}	2.5×10^{-3} Cd^{++}	0.64	35
0	2.5×10^{-3} Mg^{++}	1.00	—
10^{-2}	2.5×10^{-3} Mg^{++}	0.43	0

The enzyme was added to an assay mixture containing 1 M Tris, *p*H 8.0, 10^{-3} M *p*-nitrophenyl phosphate, and cyanide as specified to give an enzyme concentration of 1.2×10^{-8} M. Metal ions were added after the addition of enzyme. V_i is the activity of the inhibited enzyme, V_c is that of the control.

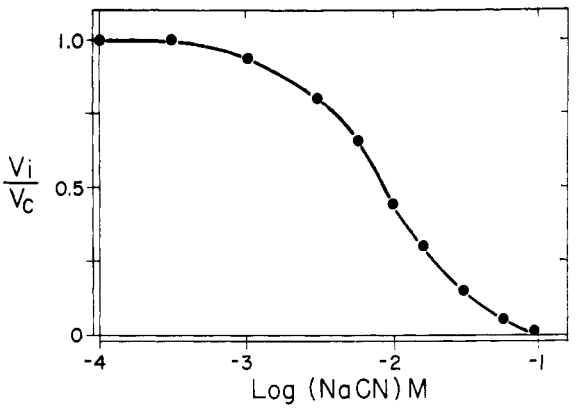

Fig. 3.—Instantaneous inhibition of *E. coli* alkaline phosphatase by sodium cyanide. Enzyme was added to an assay mixture containing 10^{-3} M *p*-nitrophenyl phosphate and sodium cyanide in 1 M Tris, *p*H 8.0. Enzyme concentration in assay mixture, 1.2×10^{-8} M.

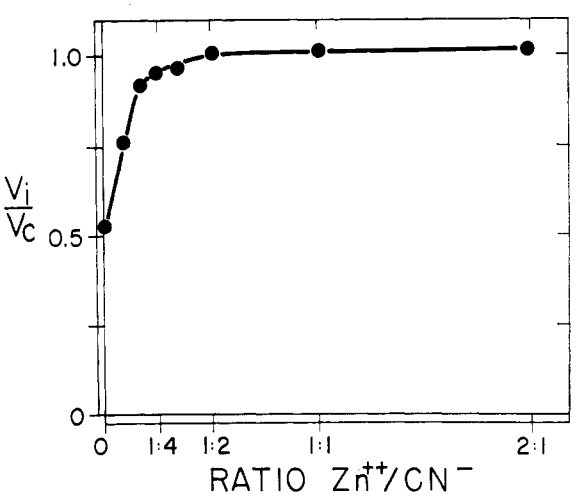

Fig. 4.—Reversal of the instantaneous inhibition of *E. coli* alkaline phosphatase with cyanide on addition of zinc ions. Enzyme was added to an assay mixture containing 10^{-3} M *p*-nitrophenyl phosphate and 10^{-2} M sodium cyanide in 1 M Tris, *p*H 8.0, 25°; enzyme concentration, 1.2×10^{-8} M. After mixing, zinc ions were added and the activity measured.

nesium is a contaminant in this system; its presence as a contaminant during the purification of other metalloenzymes is well documented (Vallee and Hoch, 1955; Vallee and Neurath, 1955).

The direct, positive correlation between increasing zinc content and increasing specific activity during purification suggests a functional role for zinc in this enzyme (Vallee, 1960). The inhibition of activity on addition of metal-binding agents to the enzyme, and the positive correlation between the order of the values of $-\log K_I$ and $\log K_{Zn}$ for three of these agents, support this hypothesis. A similar correlation has recently been reported for the zinc enzyme carboxypeptidase A; the data obtained on that system indicated that inhibition of activity is a direct function of the relative affinities of the metal-binding agents for zinc and that they inhibit by competing with the apoenzyme for the functionally essential zinc atom (Coombs *et al.*, in preparation). The data obtained here on *E. coli* alkaline phosphatase suggest a similar mechanism.

The instantaneous reversible inhibition of the enzyme with sodium cyanide resembles the inhibitions obtained with other zinc metalloenzymes (Vallee and Hoch, 1957; Hoch *et al.*, 1958; Adelstein and Vallee, 1959). The kinetics of such inhibitions are consistent with the formation of a dissociable enzyme-inhibitor complex:

$$E\cdot Zn + I \rightleftharpoons E\cdot Zn\cdot I$$

This mechanism readily accounts for the observed reversal of the inhibition either on dilution of the enzyme-inhibitor complex or on addition of metal ions which themselves form complexes with cyanide. Since zinc ions form 1:4 complexes with cyanide (Kolthoff and Lingane, 1952) the nearly complete reversal of inhibition obtained with this ratio of zinc to cyanide is readily explained. On this basis the cyanide complexes of cupric and cadmium ions account for the ability of these metal ions to reverse the cyanide inhibition, while magnesium ions, which do not form cyanide complexes, are not effective.

The failure of magnesium ions to affect the activity of the uninhibited enzyme is of interest, in view of the reported effect of this metal on the activity of mammalian alkaline phosphatases (Erdtmann, 1927; Cloetens, 1941; Binkley, 1961). This point has been discussed elsewhere (Plocke, 1961).

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