mer to hydrolyze homopolymers of lysine and of ornithine, in view of the report that a *S. typhimurium* strain is naturally inhibited not only by triornithine but also by trilysine (Sussman and Gilvarg, 1970).

References


Brown, J. L. (1973), *J. Biol. Chem. 248,* 409.

Fruton, J. S., and Simmonds, S. (1950), *Cold Spring Harbor Symp. Quant. Biol. 14,* 55.

Goldberg, A. J., and Dice, J. F. (1974), *Annu. Rev. Biochem. 43,* 835.

Goldberg, A. L., Howell, E. M., Li, J. B., Martel, S. B., and Prouty, W. F. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1112.

Hayman, S., Gatmaitan, J. S., and Patterson, E. K. (1974), *Biochemistry 13,* 4486.

Johnson, G. L., and Brown, J. L. (1974), *Biochim. Biophys. Acta 370,* 530.

Kessel, D., and Lubin, M. (1963), *Biochim. Biophys. Acta 71,* 656.

Mandelstam, J., and Halvorson, H. (1960), *Biochim. Biophys. Acta 40,* 43.

Matheson, A. T., and Dick, A. J. (1970), *FEBS Lett. 6,* 235.

Matheson, A. T., Dick, A. J., and Rollin, F. (1970), *Can. J. Biochem. 48,* 1292.

Matheson, A. T., and Murayama, T. (1966), *Can. J. Biochem. 44,* 1407.

Matheson, A. T., and Tatrie, B. J. (1964), *Can. J. Biochem. 42,* 95.

Meisler, N., and Simmonds, S. (1963), *J. Gen. Microbiol. 31,* 109.

Miller, C. G. (1975), *J. Bacteriol. 122,* 171.

Miller, C. G., and MacKinnon, K. (1974), *J. Bacteriol. 120,* 355.

Patterson, E. K., Gatmaitan, J. S., and Hayman, S. (1975), *Biochemistry 14,* 4261.

Payne, J. W. (1972a), *J. Gen. Microbiol. 71,* 267.

Payne, J. W. (1972b), *J. Gen. Microbiol. 71,* 281.

Shimura, Y., and Vogel, H. J. (1966), *Biochim. Biophys. Acta 118,* 396.

Simmonds, S. (1966), *J. Biol. Chem. 241,* 2502.

Simmonds, S. (1970), *Biochemistry 9,* 1.

Simmonds, S. (1972), *Pept. Transp. Bact. Mamm. Gut, Ciba Found. Symp., 1971,* 44.

Simmonds, S., and Toye, N. O. (1967), *J. Biol. Chem. 242,* 2086.

Sussman, A. J., and Gilvarg, C. (1970), *J. Biol. Chem. 245,* 6518.

Sussman, A. J., and Gilvarg, C. (1971), *Annu. Rev. Biochem. 40,* 397.

Vogt, V. M. (1970), *J. Biol. Chem. 245,* 4760.

Vonder Haar, R. A., and Umbarger, H. E. (1972), *J. Bacteriol. 112,* 142.

Wasmuth, J. J., and Umbarger, H. E. (1974), *J. Bacteriol. 117,* 29.


# Replacement of Metal in Metalloenzymes. A Lead–Alkaline Phosphatase†

E. Sabbioni,* F. Girardi, and E. Marafante

ABSTRACT: Lead ions can interact with calf intestine alkaline phosphatase. Experiments using $^{203}Pb$-labeled $Pb^{2+}$ ions showed that $Pb^{2+}$ ions bind the native protein in a molar ratio of Pb/protein of 1:5 with moderate inhibition of its biochemical activity. The 4 g-atoms of Zn per mol present in the native enzyme may be removed by dialysis against EDTA. The inactive apoenzyme is capable of incorporating $Pb^{2+}$ ions in a Pb/protein molar ratio of 2:1, giving a lead–protein complex still enzymatically active also when genetic material, such as nucleotides or DNA, has been used as a substrate. The reconstituted lead–protein is capable of binding $Zn^{2+}$ ions without any release of the $Pb^{2+}$ ions and with an increase in the catalytic activity of only 10–15%. The absence of Zn in the inactive apoenzyme as well as in the reconstituted lead–protein, the incorporation of $Pb^{2+}$ ions in stoichiometric amounts in the apoenzyme, and the weak influence of the $Zn^{2+}$ ions on the enzymatic assay of the lead–enzyme suggest that lead ions partially reactivate the calf intestine alkaline phosphatase apoenzyme.

It has been found that Zn atoms can be removed from the *Escherichia coli* alkaline phosphatase with a consequent complete loss of the enzymatic activity (Lazdunski and Lazdunski, 1969). The obtained apoenzyme is still capable of incorporating divalent metals such as Zn, Co, Cd, Ni, Mn, Hg, and Cu. However, only Zn- and Co-alkaline phosphatase were found to be significantly active enzymes, while the other metalloproteins show a negligible catalytic activity (Plocke and Vallee, 1962). Limited studies have been carried out on the replacement of Zn with other metal ions in mammalian alkaline phosphatase (Thoai et al., 1947; Harkness, 1967). In particular, we have found nothing in the literature dealing with the interaction of lead with mammalian alkaline phosphatase, although Kosmider established a relationship between the toxic effect of lead and the diminished activity of the enzyme in experimental animals and man (Kosmider, 1963).

In this paper the replacement of the native metal constituent (Zn) by lead in calf intestine alkaline phosphatase resulted in an enzymatically active Pb-enzyme. The study

† From the Chemistry Department (E.S. and F.G.) and the Biology Group, D.G. Research, Science and Education (E.M.), EURATOM Joint Research Center, Ispra, Italy. *Received July 22, 1975.*

was performed using nucleotides and DNA as substrates because it has been suggested that mammalian alkaline phosphatase, which can hydrolyze nucleotide mono-, di-, and triphosphate esters, could be involved in the regulation of the level of free nucleotides (Georgatsos, 1967) or in the control of the polymerization of nucleic acids in the cell (Cox and Griffin, 1965).

These experiments required highly sensitive radiochemical techniques such as radiotracing with carrier-free $^{203}Pb$ to give direct evidence of an effective incorporation of $Pb^{2+}$ ions in the inactive apoenzyme and neutron activation analysis of the end phosphate from DNA to determine the biochemical activity using single-stranded calf-thymus DNA as a substrate.

Our experiments showed that $Pb^{2+}$ ions can interact with the enzyme by different mechanisms. $Pb^{2+}$ ions bind both the native Zn-enzyme with consequent moderate inhibition of its enzymatic activity and the inactive Zn-free apoenzyme leading to a partially active Pb-enzyme.

The data obtained suggest that the restoration of the biochemical activity of the lead-enzyme is really due to the interaction of lead ions with inactive apophosphatase. A few properties of the Pb-enzyme are also compared with those of the native protein.

## Experimental Section

*Enzyme and Substrate.* All vessels used were washed in acid. Calf intestine alkaline phosphatase, degree of purity I, was supplied by Boheringer (Mannheim, Germany). The suspension of the enzyme (5 mg/ml) was centrifuged for 15 min at 30000 *g*; the precipitate was dissolved in 1 ml of 50 m*M* Tris-acetate buffer (pH 8.2) and dialyzed for 24 hr at 4°C against several changes of the same buffer. The protein was purified using DEAE-cellulose chromatography, according to Fosset et al. (1974), by eluting the enzyme on a DEAE column with 0.1 *M* Tris-acetate at pH 8.5. The catalytic activity of the purified enzyme was determined according to Garen and Levinthal (1960). For calculation of the protein concentration an $E_{1\%}^{1cm} = 7.6$ at 278 nm was assumed.

Ribo- and deoxyribonucleotides were products of Boehringer and *p*-nitrophenyl phosphate was from Sigma Chemical Co.

A calf-thymus DNA solution (3 mg/ml) (Boheringer) was dialyzed for 24 hr at 4°C against several changes of 50 m*M* Tris-acetate buffer (pH 8). After dialysis the solution of DNA was denatured by heating it to 98°C and then rapidly cooling it in an ice bath. The concentration of single-stranded DNA was calculated by determining the total nucleotide phosphorus by neutron activation analysis (Sabbioni et al., 1971). Solutions were adjusted to values of 1 mg of nucleic acid/ml. The molecular weight of the DNA was determined by end-phosphate neutron activation analysis which gave the ratio of total phosphate to terminal phosphate enzymatically released (Clerici et al., 1973).

*Chemicals and Radiochemicals.* $^{14}C$-Labeled nucleotide 5′-monophosphate and [2-$^{14}C$]EDTA were purchased from Radiochemical Center (Amersham, U.K.) with a specific activity of about 400 mCi/mmol. $^{203}Pb$ and $^{65}Zn$ were prepared carrier-free by proton irradiations of metallic thallium and copper targets in the cyclotron of the University of Milan and purified by solvent extraction and ion exchange chromatography (Girardi et al., 1969). Stable $Pb(NO_3)_2$, $ZnCl_2$, and $(NH_4)_2IrCl_6$ were supplied by Johnson-Mattey Chemicals (London). Chelex-100 resin, 200–400 mesh (analytical grade), was obtained from Bio-Rad Laboratories; DEAE-cellulose was from Pharmacia (Uppsala, Siveden); γ-aluminum oxide was from B.D.H. Trizma was a product of Sigma Chemical Co. and $KHCO_3$ was from C. ERBA.

*Metal Content of Enzymes and Buffers.* The metal content of microsamples of native alkaline phosphatase apoenzyme and reconstituted lead-protein was determined by neutron activation analysis. Samples of 300–500 μg of proteins in a 10 m*M* $KHCO_3$ buffer (pH 8.2) were lyophilized and sealed in silica vials. Standards containing 1–5 μg of each element as well as blank samples were irradiated and processed in a similar way. The metal contents of Tris and bicarbonate buffers were determined by the same technique. Irradiations were carried out in a thermal neutron flux of $10^{13}$ neutrons $cm^{-2}$ $sec^{-1}$ in a Triga Mark II reactor (LENA Lab., Pavia, Italy). The neutron activated samples were mineralized by wet-ash digestion (Sabbioni et al., 1971); the induced radioisotopes were radiochemically separated by distillation or ion-exchange chromatography and measured by γ-ray spectroscopy or Cerenkov counting (Pietra et al., 1974, 1975). Lead, for which neutron activation analysis has a low sensitivity, was determined in the native enzyme by atomic absorption spectrometry using a graphite furnace (Perkin-Elmer HCA 70) at a wavelength of 2833 Å.

*Radioactivity Measurements.* The $^{14}C$ radioactivity was measured in a liquid scintillation counter (Intertechnique apparatus Model SL 30). Samples (0.5 ml) were added to 10 ml of the liquid scintillator (Dimilume) in polyethylene vials. $^{65}Zn$ and $^{203}Pb$ radioactivities were measured by computer-coupled γ-ray spectrometry by means of a Ge(Li) detector at the characteristic lines of 1115 and 279 keV, respectively, or by Cerenkov counting (Guzzi et al., 1974).

*Cleavage of End Phosphate from Nucleotides and DNA.* The enzymatic hydrolysis of the terminal phosphate of mononucleotides was followed by a radioenzymatic assay using $^{14}C$-labeled nucleotides as the substrate. After the enzymatic reaction:

$$[^{14}]\text{nucleotide-P}_i \xrightarrow{\text{enzyme}} [^{14}C]\text{nucleoside} + P_i$$

the determination of the labeled nucleoside formed was used to measure the reaction rate after its rapid isolation from the unreacted [$^{14}C$]nucleotide by a column of γ-aluminum oxide. The assays were performed at pH 8.2 and 37°C under the following conditions: 10 m*M* $KHCO_3$ or 1 *M* Tris-acetate buffer, 10 m*M* stable nucleotide, plus 0.08 μCi of [$^{14}C$]nucleotide. The reactions were started by the addition of 0.1 μg of the enzyme to the above assay mixture (final volume 0.5 ml). After 5 min of incubation the reaction was stopped by adding 0.1 ml of a solution of 10 m*M* $(NH_4)_2IrCl_6$ (Sabbioni and Marafante, 1975).

The $^{14}C$ radioactivity of the nucleoside formed was determined in the eluate of the aluminum oxide column (0.8 × 2 cm) on which the reaction mixture was absorbed. By this method all the measurements of the reaction rate were calculated on the basis of a change of only 4–5% in the substrate concentration. The enzymatic cleavage of the terminal phosphate of single-stranded DNA was measured by end-phosphate neutron activation analysis of polynucleotides (Clerici et al., 1973).

The assay reaction mixtures contained: DNA, 1 mg/ml; Tris-acetate buffer (pH 8.2), 1 *M*; the enzyme, 0.5 μg/ml in a final volume of 0.8 ml at 37°C. The reactions were stopped at 1-min intervals of time when the enzymatically

Table I: In Vitro Preparation of $^{203}Pb^{2+}$- and $^{65}Zn^{2+}$-Labeled Calf Intestine Alkaline Phosphatases.

| Sample | Before Chelex Separation, Sp Act. of Protein[b] | After Chelex Separation: Sp Act. of Protein[b] | After Chelex Separation: μg of Pb Incorpd | After Chelex Separation: g-atom of Pb/mol of Protein[c] |
|---|---|---|---|---|
| Native enzyme | 410 | 398 | | 0.05[d] |
| Native enzyme + 5 m*M* $^{203}Pb$-labeled $Pb^{2+}$ | 335 | 330 | 0.178 | 0.2 |
| Apoenzyme[a] | 0.1 | 0.08 | | |
| Apoenzyme + 5 m*M* $^{203}Pb$-labeled $Pb^{2+}$ | 200 | 196 | 1.76 | 2 |
| Blank (buffer + 5 m*M* $^{203}Pb$-labeled $Pb^{2+}$ | | | 0.0083 | |
| | Sp Act. of Protein[b] | Sp Act. of Protein[b] | μg of Zn Incorpd | g-atom of Zn/mol of Protein[c] |
| Native enzyme | 410 | 400 | | 4[e] |
| Native enzyme + 5 m*M* $^{65}Zn$-labeled $Zn^{2+}$ | 396 | 352 | 0.001 | 4 |
| Apoenzyme | 0.1 | 0.1 | | 0.04 |
| Aponezyme + 5 m*M* $^{65}Zn$-labeled $Zn^{2+}$ | 340 | 338 | 2.1 | 4 |
| Blank (buffer + 5 m*M* $^{65}Zn$-labeled $Zn^{2+}$ | | | | |

[a] The apoenzyme was obtained by incubation of the native intestinal alkaline phosphatase with 50 m*M* EDTA (pH 6.5) at 25°C for 30 min and dialysis against 50 m*M* Tris-HCl buffer. Labeled metallophosphatases were obtained by reconstitution on addition of 5 m*M* $Zn^{2+}$ labeled with 100 μCi of $^{65}Zn$ or 5 m*M* $Pb^{2+}$ labeled with 1 mCi of $^{203}Pb$ to 600 μg of apoenzyme in a 0.5 *M* Tris-HCl buffer (pH 8.2). The incubation mixture was passed through a Chelex-100 column; the excess of labeled metal ions were fixed by the column while $^{65}Zn^{2+}$ or $^{203}Pb^{2+}$ reactivated apoenzyme was present in the eluate. The amount of metal bound to the apoenzyme was calculated by measuring $^{65}Zn$ or $^{203}Pb$ radioactivity in the eluate. [b] All enzy matic assays were carried out according to Garen and Levinthal (1960). [c] The molecular weight of the native protein (140000) was obtained from the data of Fosset et al. (1974). [d] Determined by atomic absorption. [e] Determined by neutron activation analysis.

Table II: Effect of $Zn^{2+}$ and $Pb^{2+}$ Ions upon the Metal Constituent of the $Zn^{2+}$- and $Pb^{2+}$-Alkaline Phosphatases.[a]

| Sample | Before Chelex Separation, Sp Act. of Protein[b] | After Chelex Separation: Sp Act. of Protein[b] | After Chelex Separation: % Radioact. in the Eluate, $^{65}Zn$ | After Chelex Separation: % Radioact. in the Eluate, $^{203}Pb$ |
|---|---|---|---|---|
| [$^{65}Zn^{2+}$]Phosphatase | 338 | 330 | 98 | |
| [$^{65}Zn^{2+}$]Phosphatase + 5 m*M* $^{203}Pb$-labeled $Pb^{2+}$ | 330 | 326 | 98 | 0.06 |
| Blank (buffer + 5 m*M* $^{203}Pb$-labeled $^{203}Pb^{2+}$ | | | | 0.001 |
| [$^{203}Pb^{2+}$]Phosphatase | 196 | 192 | | 99 |
| [$^{203}Pb^{2+}$]Phosphatase + 5 m*M* $^{65}Zn$-labeled $Zn^{2+}$ | 196 | 224 | 0.01 | 99 |
| Blank (buffer + 5 m*M* $^{65}Zn$-labeled $Zn^{2+}$ | | | 0.0003 | |

[a] $^{65}Zn^{2+}$- and $^{203}Pb^{2+}$-labeled calf intestine phosphatases were prepared as described in the Experimental Section. [$^{65}Zn^{2+}$]- or [$^{203}Pb^{2+}$]phosphatases (200 μg) were incubated with 5 m*M* $Pb^{2+}$ plus 1 mCi of $^{203}Pb^{2+}$ and 5 m*M* $Zn^{2+}$ plus 1 mCi of $^{65}Zn^{2+}$, respectively. The mixture of incubation was passed through a Chelex-100 column. The eluate from the column was counted for measuring $^{65}Zn$ and $^{203}Pb$ radioactivities bound to the protein, while all labeled metal ions were fixed by the column. [b] All assays were performed according to Garen and Levinthal (1960) using *p*-nitrophenyl phosphate as substrate.

released terminal phosphate was determined.

*Preparation of $^{65}Zn^{2+}$- and $^{203}Pb^{2+}$-Labeled Calf Intestine Alkaline Phosphatases.* The native calf intestine phosphatase (5 mg/ml) purified on the DEAE column was incubated with 50 m*M* EDTA plus 1 μCi of [2-$^{14}$C]EDTA in 50 m*M* Tris-HCl buffer (pH 6.5) at 25°C for 30 min. The solution was dialyzed for 30 hr at 4°C against several changes of the same buffer to remove the EDTA complexes. The radioactivity of $^{14}C$ in the protein fraction (apophosphatase) was less than 0.02%. The $^{203}Pb^{2+}$-labeled enzyme was prepared by incubating 600 μg of both of the apoenzymes, completely devoid of enzymatic activity (see Table I) and of the native protein with 5 m*M* $Pb(NO_3)_2$ plus 1 mCi of carrier-free $^{203}Pb^{2+}$ at 37°C for 30 min in 0.5 *M* Tris-HCl buffer (pH 8.2). The excess $^{203}Pb^{2+}$ ions were removed from the $^{203}Pb$-protein complex on a 1 × 2.5 cm Chelex column equilibrated with 1 *M* Tris-HCl buffer; the $^{203}Pb^{2+}$ ions were fixed by the resin. The $^{65}Zn^{2+}$-labeled enzyme was prepared in the same way.

*Effect of $^{65}Zn^{2+}$ and $^{203}Pb^{2+}$ Ions on the Metal Constituent of [$^{203}Pb^{2+}$]- and [$^{65}Zn^{2+}$]phosphatases.* $^{65}Zn^{2+}$- and $^{203}Pb^{2+}$-labeled enzymes (200 μg) were each incubated in 1 *M* Tris-HCl buffer with 5 m*M* $Pb(NO_3)$ plus 1 mCi of $^{203}Pb^{2+}$ and 5 m*M* $ZnCl_2$ plus 1 mCi of $^{65}Zn^{2+}$ to provide information on the incorporation and release of constituent metal ions of the proteins. The excess metal ions were removed on a Chelex column (0.8 × 2.5 cm) as previously described. The eluates from the column were measured for $^{65}Zn$ and $^{203}Pb$ radioactivities. The biochemical activity of the proteins in the eluate was measured using *p*-nitrophenyl phosphate as a substrate as described in the section on Enzyme and Substrate.

*Effects of Metals, pH, and Buffer Concentration on the Catalytic Activity of [$Zn^{2+}$]- and [$Pb^{2+}$]-Phosphatases.* The effect of metals upon the catalytic activity of $Zn^{2+}$- and $Pb^{2+}$-alkaline phosphatases was tested by incubating a mixture of 10 m*M* $KHCO_3$ buffer (pH 8.2), 10 m*M* dAMP plus 0.08 μCi of [$^{14}$C]dAMP, $10^{-5}$ *M* of each metal with 0.3 μg of the enzyme in a final volume of 1.5 ml for 5 min at 37°C. The reaction was stopped by adding cold 1 *M* acetate buffer up to a final pH of 4. The samples were processed on a γ-aluminum oxide column to separate the [$^{14}$C]nucleoside formed from the unreacted [$^{14}$C]nucleotide as described above. In a similar way the effect of the lead concentration upon the catalytic activity was also determined. The radioenzymatic assay was also used to determine the effect of pH and buffer concentrations upon the enzymatic activity of $Zn^{2+}$- and $Pb^{2+}$-phosphatases by incubating 0.16 μg of protein with 10 m*M* [$^{14}$C]dAMP at different pH values or concentrations of Tris-HCl and $KHCO_3$.

## Results

Table I shows that the chromatographically purified native protein has a specific activity of 410 units (micromoles of *p*-nitrophenyl phosphate hydrolyzed for 1 min with 1 mg of enzyme at pH 8 in 1 *M* Tris-HCl buffer at 37°C). The same table illustrates the results of the preparation of $^{65}Zn^{2+}$- and $^{203}Pb^{2+}$-labeled calf intestine phosphatases from the native protein.

Table II illustrates the effect of $Zn^{2+}$ and $Pb^{2+}$ ions upon

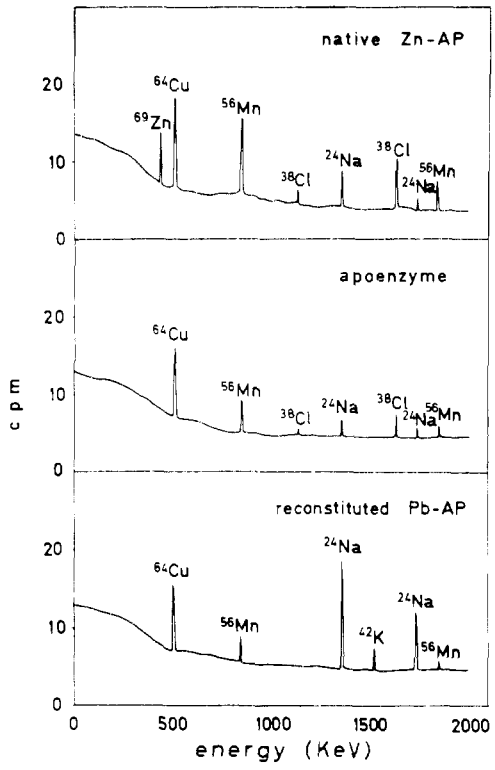


FIGURE 1: The γ-ray spectrum of neutron activated native calf intestine phosphatase, apoenzyme, and reconstituted $Pb^{2+}$-phosphatase. Proteins (300–500 μg) were irradiated in a neutron thermal flux of $10^{13}$ neutrons $cm^{-2}$ $sec^{-1}$. The γ-ray spectrum was obtained by high-resolution computer coupled γ-ray spectrometry by means of a 40 $cm^3$ Ge(Li) detector.

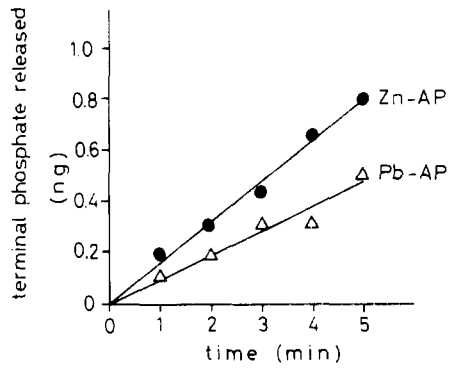


FIGURE 2: The enzymatic hydrolysis of the terminal phosphate of a single-stranded DNA by native calf intestine phosphatase and reconstituted $Pb^{2+}$-phosphatase.

the metal constituent of the $Pb^{2+}$- and $Zn^{2+}$-phosphatases, respectively. From the data reported in Tables I and II the following conclusions can be drawn: (1) while the native protein does not bind a significant amount of additional $^{65}Zn^{2+}$ ions, it can bind $^{203}Pb^{2+}$ ions in a Pb/protein molar ratio of 1:5 without significant change in the enzymatic activity; (2) the inactive apoenzyme binds $^{65}Zn^{2+}$ ions in a Zn/protein molar ratio of 4:1 to give a [$^{65}Zn^{2+}$]phosphatase with a recovery of 85–90% of the original activity of the native protein; (3) the inactive apoprotein was capable of binding $^{203}Pb^{2+}$ ions in a Pb/protein molar ratio of 2:1 to give a [$^{203}Pb^{2+}$]phosphatase with a recovery of about 50% of the biochemical activity of the native enzyme; (4) the reconstituted $Pb^{2+}$-alkaline phosphatase was able to bind $^{65}Zn^{2+}$ ions in a Zn/enzyme molar ratio of 2:5; (5) $^{203}Pb$ and $^{65}Zn$ atoms were not removed from the respectively labeled phosphatases when the proteins were incubated with an excess of labeled $^{65}Zn^{2+}$ and $^{203}Pb^{2+}$ ions, respectively.

Figure 1 illustrates a typical γ-ray spectrum of neutron activated native enzyme, apoenzyme, and reconstituted $Pb^{2+}$-phosphatase. It may be seen that Zn, present in the native protein, is practically absent in the apoenzyme and $Pb^{2+}$-phosphatase.

The Zn content of the native phosphatase samples ranged from 1774 to 2007 μg/g of protein with an average of 1890 μg/g or 4.05 g-atoms/140000. The copper content ranged from 318 to 408 μg/g of protein with an average of 363 μg/g or 0.8 g-atom/140000. About 0.8 g-atom of iron and 0.9 g-atom of phosphorus/mol of protein were also found in the native protein. The apoenzyme and $Pb^{2+}$-phosphatase contained less than 0.03 mol of phosphorus/mol of protein. As, Au, Cd, Co, Hg, Mg, Mn, and Ni were present in quantities much less than 0.08 g-atom of each metal/140000. Neutron activation analysis of $KHCO_3$ and Tris buffers used in the study revealed the presence of 0.1 and 370 ppb of Zn, respectively. As, Au, Co, Cs, Cu, Fe, Hg, Mn, and P were present only at the parts per billion level.

The relative reaction rates, in different buffers, for the native phosphatase, apoenzyme, and reconstituted $Zn^{2+}$- and $Pb^{2+}$-phosphatases showed that the $Zn^{2+}$-protein recovered in our cases was 85–90% of the original activity of the native enzyme while the $Pb^{2+}$-protein recovered 30% in $KHCO_3$ buffer and 50% in Tris buffer.

The optimal activities for the native protein and reconstituted $Pb^{2+}$-phosphatase were obtained at pH 8.5 and 8.6, respectively. The maximum reaction rate occurs using 1.5 *M* Tris-HCl or 10–50 m*M* $KHCO_3$ buffer. Figure 2 shows the reaction rate of the enzymatic hydrolysis of terminal phosphate from single-stranded DNA for the native protein and $Pb^{2+}$-phosphatase. Similar results were obtained using $^{14}C$-labeled ribo- and deoxyribonucleotide 5′-monophosphate (Table III). Adenine and cytosine were found to be the most active substrates among the nucleotides tested. Strong inhibitors of the $Zn^{2+}$-phosphatase such as $As^{5+}$, $Be^{2+}$, $Bi^{3+}$, $Ir^{4+}$, $Sb^{5+}$, $V^{5+}$, and $Zr^{4+}$ (Sabbioni et al., 1972) were also inhibitors of the $Pb^{2+}$-phosphatase.

Figure 3 shows the effect of different concentrations of lead for the two metalloproteins. No appreciable difference was observed. At a concentration of $10^{-5}$ *M* of $Pb^{2+}$ ions no inhibitory effect was observed, while for a concentration 500-fold higher about 20% inhibition was found.

## Discussion

A systematic study on the restoration of the enzymatic activity of the inactive *E. coli* alkaline phosphatase apoenzyme showed that among the metals tested (Zn, Co, Cd, Cu, Fe, Mg, Mn, and Ni) only $Zn^{2+}$ and $Co^{2+}$ partially restore the biochemical activity (Plocke and Vallee, 1962). The mammalian enzyme shows substantial differences in comparison to the *E. coli* enzyme (higher molecular weight and presence of sugars (Fosset et al., 1974), while the apoprotein from intestinal phosphatase of the bacterial protein has been reactivated by $Zn^{2+}$ ions, while $Ni^{2+}$-, $Mn^{2+}$-, and $Cu^{2+}$-phosphatases show a negligible catalytic activity (Sabbioni et al., 1972). This might imply that the metal binding sites are similar for the alkaline phosphatases from bacterial or mammalian origins.

Experiments on the calf intestine alkaline phosphatase also made possible the preparation of a lead-phosphatase still enzymatically active even when genetic material has

Table III: Relative Reaction Rates of Enzymatic Hydrolysis of $^{14}$C-Labeled Nucleotides by $Zn^{2+}$- and $Pb^{2+}$-Alkaline Phosphatase.

| Nucleotide, $10^{-2}$ M | Relative Reaction Rate[a] | |
|---|---|---|
| | $Zn^{2+}$-Phosphatase | $Pb^{2+}$-Phosphatase |
| AMP | 100 | 35 |
| CMP | 111 | 36 |
| UMP | 50 | 19 |
| GMP | 76 | 23 |
| dAMP | 106 | 40 |
| dCMP | 125 | 40 |
| dTMP | 61 | 20 |
| dGMP | 70 | 26 |

[a] All rates are referred to as relative to the enzymatic hydrolysis of AMP = 100. dAMP (10 m*M*) plus 0.08 $\mu$Ci of [$^{14}$C]dAMP were incubated with 0.1 $\mu$g of $Zn^{2+}$- or $Pb^{2+}$-phosphatase (final volume 0.5 ml). After 5 min the [$^{14}$C]nucleoside formed was isolated from the unreacted [$^{14}$C]nucleotide by a column of $\gamma$-aluminum oxide. The determination of the [$^{14}$C]nucleoside formed has been used to measure the reaction rate.

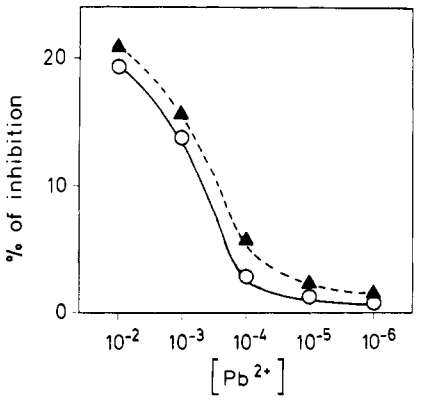


FIGURE 3: The effect of different concentrations of lead on native calf intestine alkaline phosphatase and reconstituted $Pb^{2+}$-phosphatase.

been used as the substrate.

The most important experimental difficulty in the reconstitution of $Pb^{2+}$-phosphatase from the apoenzyme was preventing the formation of lead hydroxide at pH 8. Fresh Tris buffer must be used. In this condition a lead–Tris complex is formed which is able to exchange $^{203}$Pb atoms with apoenzyme, probably in a similar manner to exchange of Zn (Plocke and Vallee, 1962). While Csopak obtained the inactive apoenzyme by batch equilibration for long periods (2–3 hr) (Csopak, 1969), in our experiments the Zn content and the enzymatic activity of the native protein were unaffected by the rapid separation on a Chelex column (Table I). In these conditions inorganic $Zn^{2+}$ and $Pb^{2+}$ ions were fixed by the resin with a decontamination factor of about $10^8$.

The following results suggest that the partial biochemical activity of $Pb^{2+}$-phosphatase was not due to experimental conditions employed for the restoration of activity: (1) $^{203}Pb^{2+}$ ions were incorporated in stoichiometric amounts into the inactive apoenzyme; the four Zn atoms of the native protein were substituted by two Pb atoms in the $Pb^{2+}$-phosphatase obtained by reconstitution; (2) the native protein was also able to bind $^{203}Pb^{2+}$ ions but in a Pb/protein molar ratio of 1:5 without appreciable change in its enzymatic activity; this means that there is a different binding site for $Pb^{2+}$ ions in the Zn-free apoenzyme in respect to the native $Zn^{2+}$ enzyme; (3) Zn, which is necessary for the catalytic function of the native protein (Riordan and Vallee, 1974), and for which the apoenzyme has a high affinity, was practically absent in the inactive apoenzyme as well as in the reconstituted $Pb^{2+}$-phosphatase; (4) the [$^{203}Pb^{2+}$]phosphatase was able to bind only 0.4 g-atom of zinc/140000 when incubated with a large excess of $^{65}Zn^{2+}$ ions (enzyme/$Zn^{2+}$ ions molar ratio, 1:3500) without any release of $^{203}Pb^{2+}$ ions from the protein. The enzymatic activity of the $Pb^{2+}$ enzyme was increased by only 10–15% (Table III).

The partial reactivation of the intestinal alkaline phosphatase apoenzyme by $Pb^{2+}$ ions may be justified by the incorporation of two lead atoms in place of the original four zinc atoms. Changes in the steric configuration of the $Pb^{2+}$-phosphatase due to the accommodation of the larger lead ions may explain the apparent impossibility of the lead enzyme of binding a stoichiometric amount of additional zinc ions higher than 0.4 g-atom of zinc/140000. Structural studies would be needed to clarify this point.

While calf intestine Co-reactivated apoenzyme does not seem to be as stable as the native enzyme, preparations of Pb-alkaline phosphatase conserve their biochemical activity after storage for 6 months at 4°C. The $Pb^{2+}$-phosphatase showed the highest catalytic activity in Tris-HCl in comparison to that obtained in $KHCO_3$ buffer. Purification of Tris-HCl by the dithiazone method (Kägi and Vallee, 1958) gave the same results. This could depend on the acceptor capability of the buffer as reported for the *E. coli* enzyme (Stadman et al., 1961; McComb and Bowers, 1972; Aw, 1975).

The effects of different metal ions at concentrations of $10^{-5}$ *M* upon the alkaline phosphatase activity were substantially the same for the native enzyme and $Pb^{2+}$-phosphatase. Strong inhibitors such as $As^{5+}$, $Ir^{4+}$, $Sb^{5+}$, $V^{5+}$, and $Zr^{4+}$ in addition to the known $Be^{2+}$ and $Bi^{3+}$ (Guilbault et al., 1969) were found. It was also found that iridium as hexachloroiridate strongly inhibits the enzymatic activity when single-stranded DNA is used as a substrate (Sabbioni and Marafante, 1975). A moderate inhibition of enzymatic activity was found for lead ions in concentrations higher than $10^{-3}$ *M*.

The neutron activation analysis of purified native calf intestine alkaline phosphatase showed the Zn-enzyme nature of the protein. However, copper, iron, and phosphorus were also found in stoichiometric amounts. The significance of the copper and iron content is unknown at present. Simpson observed Ca and Fe in bacterial alkaline phosphatase (Simpson and Vallee, 1968). Copper was not removed from the native phosphatase by EDTA treatment and it was also found in the apoenzyme and $Pb^{2+}$-phosphatase.

Recently, Bosron et al. (1975) showed that the *E. coli* alkaline phosphatase contained 1.3 g-atoms of magnesium/phosphatase mol wt 89000 suggesting that this metal can regulate the mode of zinc binding. The intestinal alkaline phosphatase in our experiments did not contain stoichiometric amounts of magnesium (Pietra et al., 1975).

It was found that phosphorus was absent in the apoenzyme and this suggests that it is bound to the active site of the native calf intestine enzyme in a similar way to that of the *E. coli* enzyme (Chappelet-Tordo et al., 1974). No significant difference in the effect of pH or buffer concentrations was observed for the $Zn^{2+}$- and $Pb^{2+}$-phosphatases. The significant reactivation of the mammalian apophosphatase with lead cannot be explained by comparison with

$Zn^{2+}$ or $Co^{2+}$ which have similar ionic radii and can replace each other in the metalloenzymes (Vallee and Wacker, 1970).

References


Applebury, M. L., and Coleman, J. E. (1969) *J. Biol. Chem. 244,* 709.

Applebury, M. L., Johnson, B. P. and Coleman, J. E. (1970), *J. Biol. Chem. 245,* 4968.

Aw, S. E. (1975), *Clin. Chim. Acta 60,* 315.

Bosron, W. F., Kennedy, F. S., and Vallee, B. L. (1975), *Biochemistry 14,* 2275.

Chappelet-Tordo, D., Iwatsubo, M., and Lazdunski, M. (1974), *Biochemistry 13,* 3754.

Cox, R. P., and Griffin, M. J. (1965), *Lancet 2,* 1018.

Clerici, L., Sabbioni, E., Campagnari, F., Spadari, S., and Girardi, F. (1973), *Biochemistry 12,* 2887.

Cox, R. P., and Griffin, M. J. (1965), *Lancet 2,* 1018.

Csopak, H. (1969), *Eur. J. Biochem. 7,* 186.

Csopak, H. and Falk, K. (1974), *FEBS Lett. 22,* 359.

Eaton, R. H., and Moss, D. W. (1967), *Biochem. J. 105,* 1307.

Fernley, H. N., and Walker, P. G. (1967), *Biochem. J. 104,* 1011.

Fosset, M., Chappelet-Tordo, D., and Lazdunski, M. (1974), *Biochemistry 13,* 1783.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38,* 1960.

Georgatsos, J. G. (1967), *Arch. Biochem. Biophys. 121,* 619.

Girardi, F., Camera, V., and Sabbioni, E. (1969), *Radiochem. Radioanal. Lett. 2,* 195.

Guilbault, G. G., Sadar, M. H., and Zimmer, M. (1969), *Anal. Chim. Acta 44,* 361.

Guzzi, G., Pietra, R., Sabbioni, E., and Girardi, F. (1974), *J. Radioanal. Chem. 20,* 751.

Harkness, D. R. (1967), *Arch. Biochem. Biophys. 126,* 513.

Kägi, J., and Vallee, B. L. (1958), *Anal. Chem. 30,* 1951.

Kosmider, S. (1963), *Pol. Arch. Med. Wewn. 32,* 1253.

Lazdunski, C., Chappelet, D., Petitclerc, C., Leterrier, F., Douzu, P., and Lazdunski, M. (1970), *Eur. J. Biochem. 17,* 239.

Lazdunski, C., and Lazdunski, M. (1969), *Eur. J. Biochem. 7,* 294.

McComb, R. B., and Bowers, G. N. (1972), *J. Clin Chem. 18,* 97.

Moss, D. W., and Walli, A. K. (1969), *Biochim. Biophys. Acta 191,* 476.

Pietra, R., Guzzi, G., and Sabbioni, E. (1974), Report EUR 5282.

Pietra, R., Sabbioni, E., and Girardi, F. (1975), *Radiochem. Radioanal. Lett. 22,* 243.

Plocke, J. D., and Vallee, B. L. (1962), *Biochemistry 1,* 1039.

Riordan, J. F., and Vallee, B. L. (1974), *Adv. Exp. Med. Biol. 48,* 33.

Sabbioni, E., Clerici, L., Campagnari, F., Girardi, F., and Bartolini, P. (1971), *Biochemistry 10,* 4185.

Sabbioni, E., Girardi, F., and Giuliani, F. (1972), Report EUR 5060, p 358.

Sabbioni, E., and Marafante, E. (1975), *Bioinorg. Chem. 5,* 32.

Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7,* 4343.

Stadman, T. C., Boyer, P. D., and Lardy, H. V. (1961), *Enzymes, 2nd Ed., 5,* 55.

Thoai, Ng.-V., Roche, J., and Roger, M. (1947), *Biochim. Biophys. Acta 1,* 61.

Vallee, B. L. (1973), *Adv. Exp. Med. Biol. 40,* 1.

Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 5,* 203.