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Role of magnesium in *Escherichia coli* alkaline phosphatase

(metal content/metalloenzyme regulation/spectral properties/tritium exchange)

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ABSTRACT **Alkaline phosphatase of *E. coli*, isolated by procedures which do not alter its intrinsic metal content, contains 1.3 ± 0.3 g-atom of magnesium and 4.0 ± 0.2 g-atom of zinc per molecule of molecular weight 89,000. Magnesium, the role of which has been unappreciated, significantly affects the function and structure of alkaline phosphatase containing either 2 or 4 g-atom of zinc per mole. Magnesium does not activate the apoenzyme but increases the activity of the enzyme containing 2 g-atom of zinc 4.4-fold and that of the enzyme containing 4 g-atom 1.2-fold. The results obtained with enzyme in which cobalt is substituted for zinc are analogous. Moreover, the absorption and electron paramagnetic resonance spectra of cobalt phosphatases reveal the effects of magnesium on cobalt coordination geometry. Addition of magnesium changes the spectral characteristics of the apoenzyme reconstituted with 2 g-atom of cobalt from predominantly octahedral to 4- or 5-coordinate geometry. These two classes of cobalt binding sites have been associated with catalysis and structure stabilization, respectively. Therefore, magnesium controls the occupancy of the catalytic and structural binding sites and modulates the resultant enzymatic activity. Hydrogen–tritium exchange was employed to determine the effects of magnesium on the conformational stability of phosphatase. Magnesium stabilizes the dynamic structural properties, both of apophosphatase and of enzyme containing 2 g-atom of zinc, which is further stabilized by 2 more zinc atoms. The role of magnesium and other metal ions in regulatory processes, only now beginning to be explored fully, will likely emerge as an important avenue for achievement of regulatory effects in metalloenzymes.**

The importance of zinc in both catalysis and structure stabilization of *Escherichia coli* alkaline phosphatase has long been recognized (1), but the presence of stoichiometric amounts of magnesium (1, 2) has been largely ignored; hence, its role has remained both unappreciated and undefined. This study confirms the presence of stoichiometric quantities of magnesium in alkaline phosphatase and further demonstrates that this metal serves to regulate the mode of binding of zinc and increases enzymatic activities over and above those attained in the presence of zinc alone. The response of the absorption and electron paramagnetic resonance (EPR) spectra of cobalt alkaline phosphatase to magnesium delineates details of its regulatory roles in cobalt—and indirectly zinc—binding and in the consequent modulations of activity (3, ‡).

MATERIALS AND METHODS

Alkaline phosphatase was released by osmotic shock from the periplasmic space of *E. coli* cells treated with EDTA (2) or Chelex 100 (3), isolated, and purified to homogeneity by methods and procedures which prevent both loss of metals and contamination (2, 3). The Chelex 100 procedure was designed to avoid exposure of phosphatase to any soluble chelating agent, thereby eliminating potential anomalies such as those attributed to EDTA (4, 5). Protein concentration was determined from absorption at 278 nm using $A_{cm}^{1\%}$ 7.2 (2). All calculations involving molarity were based on an alkaline phosphatase molecular weight of 89,000 (6). Enzymatic activity was measured with *p*-nitrophenyl phosphate, 1 mM, in 0.02 M Veronal–0.4 M NaCl or in 1 M Tris·HCl, pH 8.0, 25°. A unit of specific activity is defined as the μmol of substrate hydrolyzed per min/mg of protein, using a molar absorptivity of 1.68×10^4 for the *p*-nitrophenolate ion at 400 nm. Buffers, glassware, and substrate were freed from contaminating metals as described previously (7). After extraction with dithizone, buffers were stored in the presence of Chelex 100, 10% v/v (8). Analyses for zinc, cobalt, and magnesium were performed by atomic absorption spectrometry (9, 10) on samples that had been ashed in an electric muffle furnace at 450° for 24 hr and then dissolved in 0.1 N HCl (7).

Apoalkaline phosphatase was prepared by the procedure of Simpson and Vallee (6) using 8-hydroxyquinoline-5-sulfonic acid; the apoenzyme contained less than 0.03 g-atom of zinc and 0.01 g-atom of magnesium. No other metals were detected. Metal solutions prepared from spectrographically pure sulfate salts (Johnson Matthey & Co.) dissolved in metal-free distilled water were used to reconstitute the apoenzyme.

Visible absorption spectra were obtained with a Cary model 14 spectrophotometer equipped with a 0–0.1 absorbance slidewire. Electron paramagnetic measurements were performed at 5°K with a Varian E-9 spectrometer (11).

The conditions and procedural details of the hydrogen–tritium experiments have been described (12).

RESULTS

Metal content of native phosphatase

E. coli strain C-90 alkaline phosphatase, isolated at pH 7.5 by osmotic shock procedures employing Chelex 100 (3), contains 1.0–1.6 g-atom of magnesium with an average of 1.3 g-atom/mole of protein. Its zinc content ranges from 3.8 to 4.2 with an average of 4.0 g-atom/mole of enzyme (Table 1, samples 1–4). the magnesium content of alkaline phosphatase, isolated from the same strain and at the same pH but using EDTA instead of Chelex 100 (2), is quite similar, varying from 1.1 to 1.7 g-atom of magnesium, averaging 1.4 g-atom/mole and from 3.6 to 4.2 g-atom of zinc with an average of 4.0 g-atom/mole of protein (Table 1, samples 5–8).

Abbreviation: EPR, electron paramagnetic resonance.

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‡ F. S. Kennedy, W. F. Bosron, R. A. Anderson, and B. L. Vallee, submitted for publication.

Table 1. Metal content of alkaline phosphatase

Sample*	Metal content (g-atom/mole)† Mg+	Zn+	Σ(Mg + Zn)
1	1.6	4.2	5.8
2	1.3	4.2	5.5
3	1.3	3.9	5.2
4	1.0	3.8	4.8
5	1.2	4.2	5.4
6	1.1	4.0	5.1
7	1.7	3.6	5.3
8	1.4	4.1	5.5
Average	1.3 ± 0.3	4.0 ± 0.2	5.3 ± 0.3

* Samples 1–4 were prepared from periplasmic extracts using osmotic shock after treatment of the cells with Chelex 100 and sucrose (3); samples 5–8, Chelex 100 was replaced by EDTA (2).

† Metal content was determined by atomic absorption of ashed samples (see *Methods and Materials*).

The metal content of different isoenzymes of the C-90 strain and of alkaline phosphatase isolated from different *E. coli* strains, e.g., K-10, C_4F_1, and CW-3747,§ is completely analogous. Thus, all alkaline phosphatases isolated under these conditions contain a total of 5.3 ± 0.3 g-atom of metal per mole, of which 4 are zinc and the remainder magnesium (Table 1).

Equilibrium binding studies

The binding of magnesium to apophosphatase depends markedly on pH and increases from 0.2 to 1.7 g-atom/mole as the pH is raised from 6.8 to 9.2‡; at pH 8.0, 1.4 g-atom is bound per mole (Table 2, sample 1). Zinc significantly affects the extent of magnesium binding. At pH 6.8, the presence of zinc increases the binding of magnesium from 0.2 to 1.3 g-atom/mole‡, while at pH 8.0 it increases it from 1.4 to 1.6 g-atom/mole‡ (Table 2, samples 1 and 4). In the absence of magnesium, dialysis of the apoenzyme against 10^{-4} M zinc results in the incorporation of 4.1 g-atom of zinc per mole (Table 2, sample 2). Using very high zinc concentrations, a second class of zinc binding sites—beyond the first 4 g-atom—can be discerned from the binding isotherm‡. Dialysis of the apoenzyme against 10^{-4} M cobalt leads to the incorporation of 5.8 g-atom of this metal (Table 2, sample 3), but the binding isotherm does not discriminate among these sites. Different stoichiometries pertain when apophosphatase is dialyzed simultaneously against magnesium and cobalt. The apoenzyme binds only 4.8 g-atom of cobalt, rather than 5.8, and only 0.9 g-atom of magnesium, rather than 1.4 (Table 2, sample 5). The replacement of 1 g-atom of cobalt by 1 g-atom of magnesium implies that cobalt can bind to the sites normally occupied by zinc as well as to those which bind magnesium. Thus, there are maximally six metal binding sites (samples 3, 4, and 5). Of these, only two bind magnesium, four are relatively specific for zinc, but all six can bind cobalt, apparently indiscriminately.

The effect of magnesium on the enzymatic activity of zinc and cobalt phosphatases

Magnesium alone does not confer catalytic activity on apoalkaline phosphatase (Table 3, sample 1), but it markedly enhances the activities of both the zinc and cobalt enzymes, as demonstrated by selective reconstitution of the apoenzyme with various combinations of these ions. The activity of phosphatase depends on its zinc or cobalt content (6) and, therefore, the effect of magnesium on activity must be explored employing various amounts of these metals. Assigning the activity of the enzyme reconstituted with magnesium and 4 g-atom of zinc as 100% (Table 3, sample 2), that of alkaline phosphatase containing 2 g-atom or 4 g-atom of zinc per mole is 12% and 80%, respectively (Table 3, samples 3 and 5); addition of magnesium raises these values to 53% and 100% (Table 3, samples 4 and 2). Similar effects are seen on adding magnesium to cobalt phosphatase. Compared to the enzyme reconstituted with magnesium plus 4 g-atom of cobalt (Table 3, sample 6), the activity of alkaline phosphatase containing 2 g-atom or 4 g-atom/mole is 32% or 74%, respectively (Table 3, samples 7 and 9), but addition of magnesium raises these values to 90% and 100% (Table 3, samples 8 and 6). Addition of excess cobalt or magnesium to this last species does not affect activity further. Thus, magnesium enhances the activity of both zinc and cobalt alkaline phosphatases containing either 2 or 4 g-atom, though most

§ These strains were a generous gift of Dr. A. Torriani, Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass.

Table 2. Equilibrium binding of magnesium, zinc, and cobalt to alkaline phosphatase

Metal added	Metal bound (g-atom/mole) Mg	Zn	Co[a]	Σ(Mg + Zn or Co)
1. Mg	1.4	0	0	1.4
2. Zn	0	4.1	0	4.1
3. Co	0	0	5.8	5.8
4. Zn, Mg	1.6	4.2	0	5.8
5. Co, Mg	0.9	0	4.8	5.7

Phosphatase, 10 μM, in Tris·HCl, 50 mM, pH 8.0, was dialyzed for 24 hr, 23°, against zinc, cobalt, and/or magnesium, 100 mM. Metal analyses were performed by atomic absorption (9, 10).

[a] Samples were dialyzed as described above except in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer.

Table 3. Specific activities of metallophosphatases

Sample	Metal added (g-atom/mole) Mg	Co	Zn	Specific activity	%
1	2.5	—	—	0	0
2	2.5	—	4	54.0 ± 2	100
3	—	—	2	6.5 ± 1	12
4	2.5	—	2	28.5 ± 1	53
5	—	—	4	43.0 ± 2	80
6	2.5	4	—	1.9 ± 0.1	100
7	—	2	—	0.6 ± 0.1	32
8	2.5	2	—	1.7 ± 0.1	90
9	—	4	—	1.4 ± 0.1	74

Apoenzyme was incubated in 10 mM Tris, pH 8.0, 25°, and metal ions were added to yield the molar ratios of metal to enzyme indicated. Zinc enzymes were assayed in 1 M Tris and cobalt enzymes in 0.02 M Veronal–0.4 M NaCl containing 1 mM *p*-nitrophenylphosphate at 25°; samples were allowed to equilibrate for 24 hr prior to assaying. A 2.5-fold molar excess of magnesium was added to apoenzyme 5 hr before the addition of the second metal species.

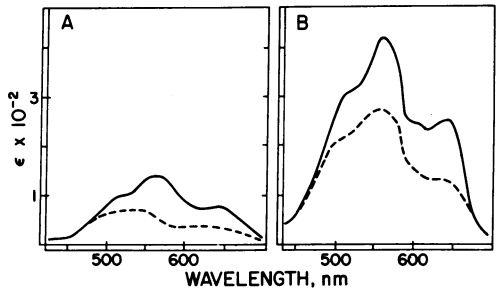

FIG. 1. Absorption spectra of cobalt phosphatases ± magnesium. (A) (- - -) 2-Co enzyme, 0.15 mM, in Tris·HCl, 10 mM, pH 8.0; (—) same except apoenzyme was preincubated with 2.5-fold molar excess of magnesium for 5 hr prior to cobalt addition. (B) Same as A, except 4 g-atom of cobalt were added to the apoenzyme.

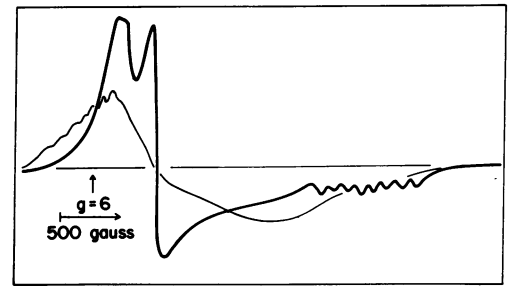

FIG. 2. Electron paramagnetic spectra of 2-Co alkaline phosphatase ± magnesium. Conditions as in Fig. 1. (Light line), 2-Co alkaline phosphatase; (heavy line) prior to the addition of 2 g-atom of cobalt the apoenzyme was preincubated for 5 hr with a 2.5-fold molar excess of magnesium. Microwave power 10 mW, modulation frequency 100 kHz, microwave frequency 9.3 GHz, 5°K.

markedly for enzymes containing only 2 g-atom of zinc or cobalt.

Effect of magnesium on the spectral properties of cobalt phosphatase

The magnesium-induced 3-fold increase in activity of the 2-Co enzyme[¶] coincides with changes in the coordination geometry of the cobalt atoms. The absorption band of the 2-Co enzyme is weak and broad with maximal absorption in the region of 510 nm, characteristic of cobalt in a predominantly octahedral environment (Fig. 1A, dashed line). The EPR spectrum is consistent with this type of geometry (Fig. 2, light line). The presence of magnesium intensifies and further defines the absorption spectrum, generating prominent maxima at 555 and 640 nm (Fig. 1A, solid line). It also generates striking changes in the EPR spectrum to result in g values of $g_1 = 4.95$, $g_2 = 3.98$, and $g_3 = 1.95$ and an average g value of 3.62 (Fig. 2, heavy line). Both the absorption and EPR spectra are consistent with cobalt in 4- or 5-coordinate sites of low symmetry.

The absorption spectrum of the 4-Co enzyme is characteristic of cobalt in an entatic environment (6); magnesium further enhances its absorptivity and spectral definition (Fig. 1B). However, in this instance there is no evidence of changes in cobalt coordination geometry. The basis of the spectral changes, whether due to local conformational or other structural effects or to redistribution of cobalt atoms among the different sites, is being established. The EPR spectra of phosphatase, containing 4 g-atom of cobalt, are complex both in the presence or absence of magnesium. Hyperfine splitting cannot be discerned and the spectra resemble those of magnetically concentrated materials. Interpretation in terms of 3 g-values has not been possible.

Effect of magnesium on the hydrogen–tritium exchange of apo- and zinc phosphatase

Reconstitution of apophosphatase with 4 g-atom of zinc per mole stabilizes the dynamic structure of the protein significantly, as evidenced by the marked additional retention of tritium (Fig. 3). The rate of tritium exchange for 2-Zn phosphatase differs from those of the apo- and 4-Zn enzymes (12). Initially, the 2-Zn enzyme retains a total number of tritium atoms intermediate between those characteristic of the apo- and holoenzymes, but as a function of time the decay curve becomes identical with that of the apoenzyme. This indicates that the 2-Zn phosphatase is less stable than the 4-Zn enzyme and may lose or site-exchange its zinc atoms, consistent with the reversible loss of enzymatic activity of this species (12). We now find that magnesium markedly stabilizes the structures both of the apoenzyme and of that containing 2 g-atom of zinc, which is then further stabilized by 2 more zinc atoms to form the 4-Zn 1.6-Mg holoenzyme (Fig. 3).

DISCUSSION

Metalloenzymes may contain one or more sets of metal binding sites whose occupancy by the same or different metal atoms critically affects catalysis, structural stability, or both. Such considerations pertain to alkaline phosphatase of *E. coli*, though universal agreement has not been reached regarding its metal content and its implications to function and structure.

The present findings help to resolve past differences regarding the metal content of alkaline phosphatase. Enzyme prepared either by the method of Simpson *et al.* (2), or of Bosron *et al.* (3), contains 1.0–1.7 g-atom of magnesium and from 3.6 to 4.2 g-atom of zinc per molecule of molecular

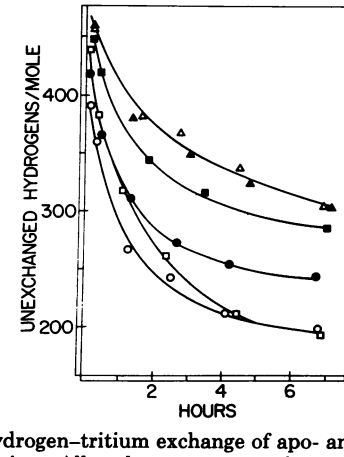

FIG. 3. Hydrogen–tritium exchange of apo- and zinc phosphatases ± magnesium. All exchanges were performed in Tris·HCl, 10 mM, pH 8.0, 4°. Protein concentrations were 10 mg/ml prior to passage over the large column and 1–2 mg/ml during subsequent incubations. (○) apophosphatase; (□) 2-Zn phosphatase; (△) 4-Zn phosphatase. Closed symbols (●, ■, ▲) represent the respective enzymes containing magnesium; under these conditions the apoenzyme binds 1.4 g-atom of magnesium and the 2- and 4-Zn enzymes bind 1.6 g-atom.

[¶] The number of metal atoms added to alkaline phosphatase is identified by an Arabic number preceding the metal symbol, e.g., 2-Zn alkaline phosphatase, 4-Zn phosphatase refer to the enzyme to which 2 and 4 g-atom of zinc have been added per mole.

weight 89,000 (Table 1). Virtually all previous reports cite between 2 and 6 g-atom of zinc per mole of enzyme (1, 2, 5, 13–15), though some regard any zinc content beyond 2 g-atom as unimportant (5, 13). Save for the instances mentioned (1, 2), the magnesium content has not been reported. It is now apparent that this element is critical both to the total zinc content and the optimal activity of phosphatase.

In general, ambiguities in metal content of enzymes can be due to variations in the analytic methodology employed for characterization, the protein molecular weight value used to determine molar stoichiometry, and the isolation conditions used to select a representative biological sample. We have recently reviewed the manner in which these considerations have affected past interpretations of data regarding the metal content of alkaline phosphatase (3). Moreover, extrapolation of the effects observed when EDTA is employed to prepare apoenzyme (4) to those presumed to occur if EDTA is utilized in the isolation procedure (5, 16) has compounded the problems created by the above procedural factors. Table 1 demonstrates that the zinc and magnesium contents of alkaline phosphatase are independent of whether EDTA or the insoluble chelating agent Chelex 100 is employed during the isolation procedure.

The present data confirm that magnesium alone does not restore activity to the apoenzyme, and they demonstrate further that magnesium and zinc affect alkaline phosphatase function and metal binding cooperatively (Tables 2 and 3). Dependent on the pH of isolation, alkaline phosphatase contains between one and two magnesium atoms which control the binding of the functional and structural zinc atoms. Zinc, in turn, affects magnesium binding. The confirmation of the cooperative magnesium–zinc effect on the activity of zinc phosphatase recognized more than a decade ago (1) should now clarify certain of the molecular features critical to the mechanism of action of this enzyme.

Failure to monitor the occurrence of magnesium in *E. coli* alkaline phosphatase has prevented recognition of its importance to the function, structure, and zinc content of the enzyme. This also likely underlies the contradictions in literature reports regarding both the metal content and stoichiometry of the native and reconstituted enzymes and their respective activities. Moreover, the marked pH-dependence of magnesium binding and the addition of large amounts of exogenous magnesium during purification of the enzyme virtually eliminated the possibility of detecting any endogenous magnesium, thus obscuring its functional and structural roles, including effects on zinc (cobalt) binding (1, 5, 15, 17–19). It is now apparent that both magnesium and zinc are involved in alkaline phosphatase catalysis and structure stabilization and mutually interact to affect their respective binding and coordination geometries. The results of equilibrium binding of various combinations of zinc, cobalt, and magnesium as a function of their concentrations, the corresponding enzymatic activities, as well as the absorption, circular dichroism, magnetic circular dichroism, and EPR spectra of the various cobalt enzymes, all support these conclusions (3, ‡).

The magnesium effects are best seen in the cobalt enzymes where changes in coordination geometry can be discerned spectrally. The visible absorption characteristics of the 2-Co enzyme, containing magnesium, suggest 4- or 5-coordinate geometry (Figs. 1 and 2), while in the absence of magnesium they indicate predominantly octahedral geometry (Figs. 1 and 2). Thus, addition of magnesium changes the cobalt coordination geometry from octahedral to 4- or 5-coordinate and increases activity 3-fold. In the case of the 4-Co enzyme there is a general increase in absorptivity (Fig. 1) which accompanies the magnesium-induced increase in activity.

These alterations of activity and spectra may reflect changes in conformation affecting coordination geometries and relative affinities of cobalt or zinc for the catalytic, structural, and regulatory sites. The oxidation of Co(II) to Co(III) in alkaline phosphatase represents an approach designed to differentiate among these sites by means of a metal probe particularly suited for this purpose (8).

Magnesium markedly enhances conformational stabilities, especially of the apo- and 2-Zn enzymes (Fig. 3), and prevents the loss of activity of the 2-Zn species (12) (Table 3). In this regard, the low value of the specific activity of the 2-Zn enzyme after equilibration for 24 hr in the absence of magnesium (Table 3, sample 2) may reflect loss of or reequilibration of zinc among different binding sites (Table 3, sample 4; Fig. 3).

Now that magnesium, zinc, histidine (20), serine (21), and arginine (22) are all implicated in alkaline phosphatase catalysis, their interrelationships and joint participation in the enzyme mechanism require examination. Since the magnesium content of most phosphatase preparations studied in the past is unknown, the kinetic properties, e.g., pH optimum, substrate, and buffer dependencies as well as the relevant catalytic constants, need to be defined with enzyme preparations of known magnesium content. Similarly, structural features such as site–site and subunit interactions deserve critical reevaluation in the light of present findings. The existence of a total of six metal binding sites per dimer and the reciprocal effects of magnesium and zinc (cobalt) on binding (Tables 2 and 3) may bear importantly on past mechanistic deductions which were based on spectra of enzymes substituted with from 2 to 4 molar equivalents of cobalt (6, 23, 24), copper (25–27), or manganese (28–30) and unknown amounts of magnesium. The effects of magnesium on alkaline phosphatase may prove to be pertinent to such features as subunit interactions, negative cooperativity (31), single-site reactivity (32), and pyrophosphatase activity (33). Similarly, the location of metal binding sites by x-ray analysis (34) will be affected by the presence of optimal amounts of magnesium and zinc in crystals employed for this purpose.

Characterization of the magnesium content and the function of this ion will likely provide important, missing links in defining the mechanism of action of alkaline phosphatase of *E. coli*. The participation of magnesium and other metal ions in regulatory processes, only now beginning to be explored fully, will likely emerge as an important avenue for the achievement of regulatory effects in metalloenzymes.

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1. Plocke, D. J., Levinthal, C. & Vallee, B. L. (1962), *Biochemistry* 1, 373–378.
2. Simpson, R. T., Vallee, B. L., and Tait, G. H. (1968), *Biochemistry* 7, 4336–4342.
3. Bosron, W. F., Kennedy, F. S. and Vallee, B. L. (1975) *Biochemistry* 14, 2275–2282.
4. Ulmer, D. D. and Vallee, B. L. (1971), *Bioinorganic Chemistry*, Advances in Chemistry Series 100, ACS, p. 193.
5. Csopak, H. and Szajn, H. (1973), *Arch. Biochem. Biophys.* 157, 374–379.

6. Simpson, R. T. and Vallee, B. L. (1968), *Biochemistry* **7**, 4343–4350.
7. Thiers, R. E. (1957), *Methods Biochem. Anal.* **5**, 273–335.
8. Anderson, R. A. and Vallee, B. L. (1975), *Proc. Nat. Acad. Sci. Usa* **72**, 394–397.
9. Fuwa, K., Pulido, P., McKay, R. and Vallee, B. L. (1964), *Anal. Chem.* **36**, 2407–2411.
10. Iida, C., Fuwa, K. and Wacker, W. E. C. (1967), *Anal. Biochem.* **18**, 18–26.
11. Kennedy, F. S., Hill, H. A. O., Kaden, T. A. and Vallee, B. L. (1972), *Biochem. Biophys. Res. Commun.* **48**, 1533–1539.
12. Brown, E. M., Ulmer, D. D. and Vallee, B. L. (1974), *Biochemistry* **13**, 5328–5334.
13. Harris, M. I. and Coleman, J. E. (1968), *J. Biol. Chem.* **243**, 5063–5073.
14. Reynolds, J. A. and Schlesinger, M. J. (1968), *Biochemistry* **7**, 2080–2085.
15. Lazdunski, C., Peticlerc, C. and Lazdunski, M. (1969), *Eur. J. Biochem.* **8**, 510–517.
16. Csopak, H., Falk, K. E. and Szajn, H. (1972), *Biochim. Biophys. Acta* **258**, 466–472.
17. Garen, A. and Levinthal, C. (1960), *Biochim. Biophys. Acta* **38**, 470–483.
18. Applebury, M. L. and Coleman, J. E. (1969), *J. Biol. Chem.* **244**, 308–318.
19. Schlesinger, M. J. and Olsen, R. (1970), *Anal. Biochem.* **36**, 86–90.
20. Tait, G. H. and Vallee, B. L. (1966), *Proc. Nat. Acad. Sci. USA* **56**, 1247–1251.
21. Schwartz, J. H. and Lipmann, F. (1961), *Proc. Nat. Acad. Sci. USA* **47**, 1996–2005.
22. Daemen, F. J. M. and Riordan, J. F. (1974), *Biochemistry* **13**, 2865–2871.
23. Applebury, M. L. and Coleman, J. E. (1969), *J. Biol. Chem.* **244**, 709–718.
24. Taylor, J. S., Lau, C. Y., Applebury, M. L. and Coleman, J. E. (1973), *J. Biol. Chem.* **248**, 6216–6220.
25. Taylor, J. S. and Coleman, J. E. (1972), *Proc. Nat. Acad. Sci. USA* **69**, 859–862.
26. Zukin, R. S. and Hollis, D. P. (1973), *J. Biol. Chem.* **249**, 656–658.
27. Csopak, H. and Falk, K. E. (1974), *Biochim. Biophys. Acta* **359**, 22–32.
28. Haffner, P. H., Goodsaid-Zulduondo, F. and Coleman, J. E. (1974), *J. Biol. Chem.* **249**, 6693–6695.
29. Zukin, R. S. and Hollis, D. P. (1975), *J. Biol. Chem.* **250**, 835–842.
30. Lilja, H., Csopak, H., Lindman, B. and Folsch, G. (1975), *Biochim. Biophys. Acta* **384**, 277–282.
31. Simpson, R. T. and Vallee, B. L. (1970), *Biochemistry* **9**, 953–958.
32. Chappelet-Tordo, D., Iwatsubo, M. and Lazdunski, M. (1974), *Biochemistry* **13**, 3754–3762.
33. Heppel, L. A., Harkness, D. and Hilmoe, R. (1962), *J. Biol. Chem.* **237**, 841–846.
34. Knox, J. R. and Wyckoff, H. W. (1973), *J. Mol. Biol.* **74**, 533–545.