87. The Calf Intestinal Alkaline Phosphatase. II. Reaction Between the Metal Content and the Enzyme Activity

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Summary

Pure alkaline phosphatase (EC 3.1.3.1; 1500 U/mg) was dialyzed at 4° during 168 h against water, 10^{-4}M EDTA or 10^{-4}M *o*-phenanthroline. During the dialysis, samples were periodically removed and analyzed for metal content and activity. The results indicate that 1 mol of native calf intestinal alkaline phosphatase contains 4 g-atom of zinc and 4 g-atom of magnesium tightly bound, and that both metal ions are necessary for full enzyme activity. The dialyzed, partially demetallized enzyme could be reactivated by the addition of zinc and/or magnesium salts.

Introduction. – There are still controversal opinions about the total number of zinc ions incorporated in the structure of the intestinal alkaline phosphatase, and especially about the exact number of these ions associated with the catalytic activity of the enzyme. *Engström* [1] found 3 g-atom, *Fosset et al.* [2], *Ensinger et al.* [3] and *Sabbioni et al.* [4] 4 g-atom of zinc per mol of enzyme. *Ensinger et al.* reported, that the intestinal alkaline phosphatase is completely inactive, if the zinc content is reduced to 2 g-atom per mol, whereas other authors [5] [6] found, that the alkaline phosphatase of *E. coli,* containing in the native state also 4 g-atom of zinc, is totally active if containing only 2 g-atom of zinc per mol. The magnesium, on the other hand, is generally considered to be more an activator and stabilizer than a structural constituent of the intestinal alkaline phosphatase [7]. In certain mammalian phosphatases, *e.g.* the pig kidney alkaline phosphatase, 2 g-atom of magnesium per mol were found [8]. We had many reasons to think that magnesium is, like zinc, tightly bound to the intestinal alkaline phosphatase and that it is a structural constituent associated with the catalytic activity. We studied therefore the relation between the activity and the metal content during a dialysis of 7 days against water and against chelators having different affinities for zinc and magnesium ions. In order to follow the gradual diminution of the metal content during the dialysis, we used very dilute chelator solutions. Finally, we studied the reactivation of the partially demetallized enzyme preparations.

Results. – The dialysis of pure calf intestinal alkaline phosphatase against quartz-distilled water at 4° during 168 h does not remove the protein bound zinc. On the other side, a great part of the magnesium present in the phosphatase

preparations is withdrawn after 24 h *(Table 1)*. The enzyme, containing at this stage of dialysis 4.5 g-atom of zinc and 4.9 g-atom of magnesium per mol of enzyme (120000 D), shows 98% of the initial activity. After a dialysis of 168 h against water, we could practically not observe any further change of the metal content, but a moderate diminution of the specific activity to 83%. These results prove that 4 sites for zinc and 4 sites for magnesium bind these metal ions so strongly, that they resist to a long-time dialysis against water. The high magnesium content found initially and during the first 24 h of dialysis indicates the presence of secondary binding sites for magnesium, which are occupied if the enzyme is isolated from magnesium-salt containing solutions. This part of magnesium ions has no or only a weak influence on the phosphatase activity.

The dialysis against a 10^{-4} M EDTA permits to remove a great deal of the protein-bound magnesium without concomitant decrease of the zinc content *(Table 1)*. In this case, the part of the magnesium bound to secondary sites is more rapidly removed. Already after a dialysis of 8 h, a molar metal content of 4.2 g-atom of zinc and of 4.0 g-atom of magnesium is reached, without significant activity change (96%). During the further dialysis (48 and 72 h), the molar zinc content remains constant, but the magnesium content is decreased to 2.4 (2.8) g-atom per mol. Parallel to this magnesium decrease, the activity falls to 64 (66) %. After a dialysis of 168 h, the molar content of magnesium was 1.2 g-atom, that of zinc 3.9 g-atom and the specific activity 39%. This shows that the zinc alone – even at 4 g-atom per mol – is not able to assure full enzymatic activity, but that 4 g-atom of tightly bound magnesium are necessary to ensure the complete activity.

On the other hand, a dialysis against 10^{-4} M *o*-phenanthroline, a strong chelator for zinc and a weak one for magnesium, removes selectively the zinc without concomitant loss of the tightly bound magnesium *(Table 1)*. After a dialysis of 168 h, the zinc content gradually decreases from 4 to 1.2 g-atom per mol, but the magnesium content remains at 3.4 g-atom. Parallel to this zinc decrease, the activity falls to about 12%, indicating the stronger dependence of the phosphatase activity on the zinc than on the magnesium content.

To get additional information concerning the influence of the metal content on the catalytic activity, we determined the V_{max} and the K_M values at different times during the dialysis of the phosphatase against 10^{-4} M EDTA and against 10^{-4} M *o*-phenanthroline. The results are listed in *Table 2*. These values show that the decrease of the magnesium content lowers only the V_{max}, whereas the decrease of the zinc content diminishes the V_{max} and the K_M values.

The dialyzed phosphatase could be easely reactivated. The enzyme preparation, dialyzed against 10^{-4} M EDTA, containing about 2 g-atom of magnesium and 3.9 g-atom of zinc and showing 56% of the initial activity *(Fig. 1)* was immediately and completely reactivated to 118% by the addition of magnesium acetate (final concentration 9.8 mM). The simultaneous addition of magnesium acetate and zinc acetate (final concentrations 9.8 mM resp. 1 μM) gave the same result, whereas the addition of zinc acetate alone (final concentration 1 μM) had no effect. But a subsequent addition of magnesium acetate resulted in a reactivation to 122%.

A phosphatase, dialyzed against *o*-phenanthroline, containing 3.4 g-atom of magnesium and 1.2 g-atom of zinc and showing about 10% of the initial activity

Table 1. *Change of the metal content and the specific activity of the calf intestinal phosphatase during a long-time dialysis*

Dialysis time [h]	External solution H_2O				10^{-4} M EDTA				10^{-4} M *o*-phenanthroline			
	Zn [g-atom/mol]	Mg [g-atom/mol]	Specific activity[a]) [U/mg]	% of initial activity	Zn [g-atom/mol]	Mg [g-atom/mol]	Specific activity[a]) [U/mg]	% of initial activity	Zn [g-atom/mol]	Mg [g-atom/mol]	Specific activity[a]) [U/mg]	% of initial activity
0	4.2	13	1310	100	4.3	12	1365	100	3.8	12	1365	100
4	4.2	10	1256	96	4.1	4.4	1338	98	4.0	9	1201	88
8	–	–	–	–	4.2	4.0	1310	96	3.6	8	901	66
24	4.5	4.9	1283	98	4.2	2.6	1010	74	3.1	5	628	46
48	4.2	4.5	1092	83	4.4	2.4	874	64	2.7	4	437	32
72	4.3	4.3	1092	83	4.6	2.8	901	66	2.0	3.5	491	36
96	4.8	4.4	1092	83	3.7	2.4	640	47	1.7	3.8	328	24
168	5.0	4.3	1092	83	3.9	1.2	533	39	1.2	3.4	164	12

[a]) 1 U = 1 μmol of substrate hydrolyzed per min at 25° and pH 9.8 (substrate: 10 mM *p*-nitrophenylphosphate; buffer 1 M 'diethanolamine'/HCl (pH 9.8) containing 5×10^{-5} M of EDTA, but no magnesium acetate).

Table 2. V_{max}- *and* K_M-*values of the calf intestinal phosphatase (substrate* p-*nitrophenylphosphate) during the dialysis against EDTA and* o-*phenanthroline* (mean of 3 determinations)

Dialysis time [h]	External solution					
	10^{-4} M EDTA[a])			10^{-4} M *o*-phenanthroline[a])		
	V_{max}/mg enzyme	%	K_M[mM]	V_{max}/mg enzyme	%	K_M[mM]
0	1442	100	0.66±0.02	1498	100	0.64±0.02
48	1230	85	0.59±0.03	927	61	0.54±0.03
96	1016	70	0.62±0.03	359	24	0.40±0.03
168	–	–	–	286	19	0.36±0.02

[a]) The corresponding zinc and magnesium values are indicated in the *Table 1.*

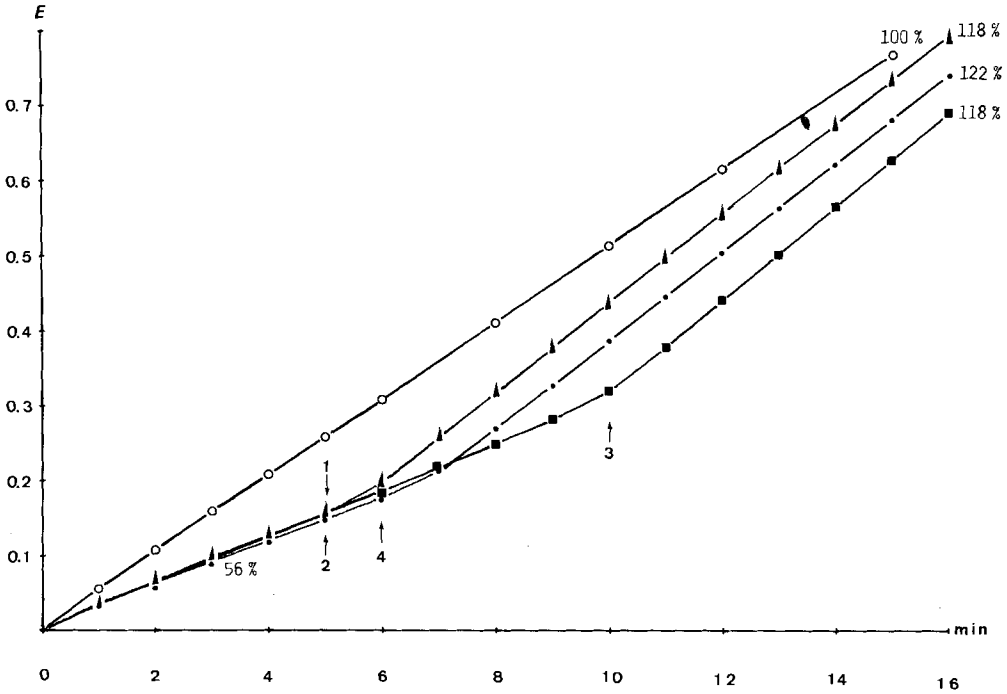

Fig. 1. *Reactivation of the phosphatase, dialyzed 96 h against* 10^{-4} M *EDTA, containing 2 g-atom of magnesium and 3.9 g-atom of zinc per mol.*

Activity of the non-dialyzed enzyme -○-○- (100%).

Activity of the dialyzed enzyme: -·-·-, -▲-▲- and -■-■- during the first 5 min of incubation (56%).

Activity after addition of $Mg(Ac)_2$ at 1↓: -▲-▲- during 5 to 16 min.

Activity after addition of $Zn(Ac)_2$ at 2↑ -■-■- during 5–10 min and after subsequent addition of $Mg(Ac)_2$ at 3↑ -■-■- during 10–16 min.

Activity after simultaneous addition of $Mg(Ac)_2$ and $Zn(Ac)_2$ at 4↑: -·-·- during 6–16 min.

ΔE/min × 2275 = activity in U/mg proteine.

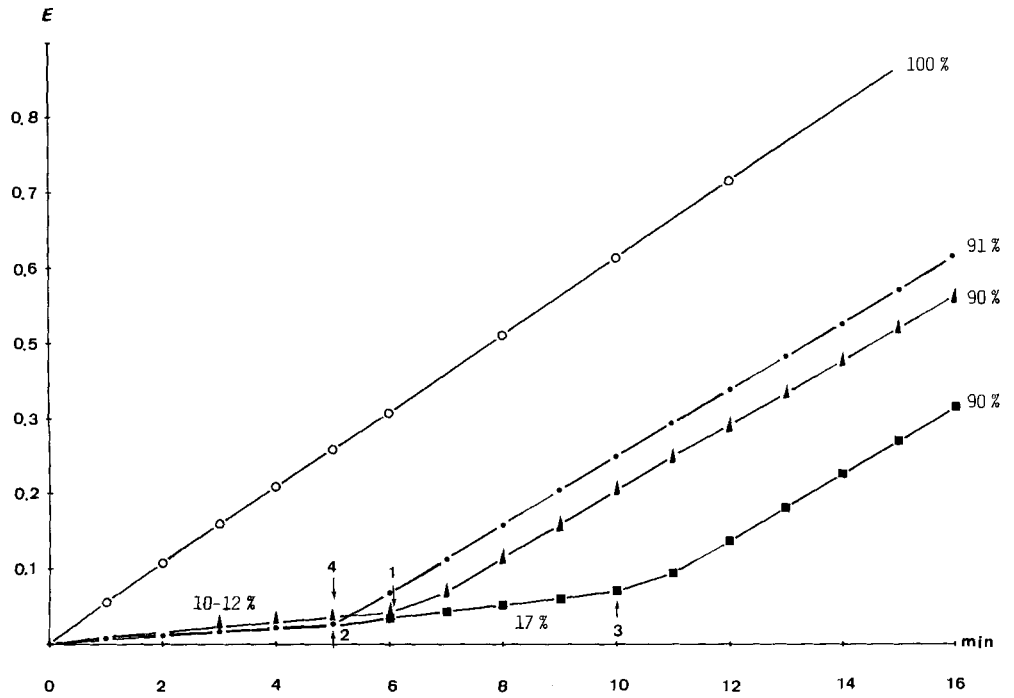

Fig. 2. *Reactivation of the phosphatase, dialyzed 168 h against 10^{-4} M o-phenanthroline, containing 1.2 g-atom of zinc and 3.4 g-atom of magnesium per mol.*
Activity of the non-dialyzed enzyme –○–○– (100%).
Activity of the dialyzed enzyme: –·–·–, –▲–▲– and –■–■– during 0 to 5 min of incubation (10–12%).
Activity after addition of $Mg(Ac)_2$ at 1↓: –▲–▲– during 6 to 16 min.
Activity after addition of $Mg(Ac)_2$ and $Zn(Ac)_2$ at 2↑: –·–·– (5–16 min).
Activity after addition of $Zn(Ac)_2$ at 4↓: –■–■– (5–10 min) and after subsequent addition of $Mg(Ac)_2$ at 3↑: –■–■– (10–16 min).
ΔE/min × 2275 = activity in U/mg proteine.

(Fig. 2) could be reactivated immediately by the simultaneous addition of magnesium acetate and zinc acetate (final concentration 9.8 mM and 1 μM) and also by the addition of magnesium acetate alone (9.8 mM). On the contrary, the addition of zinc acetate alone (1 μM) did reactivate the enzyme only from 10 to 17%, whereas magnesium acetate, added 5 min later, reactivated the enzyme to 90%.

Discussion. – It is very difficult to find a convenient technique which permits to remove separately either the zinc or the magnesium ion from the phosphatase. Moreover, these two ions are ubiquitously present as trace contaminants even in the purest chemicals used for the enzyme assay. To prevent a reactivation of the demetallized phosphatase, we measured the activity in presence of 5×10^{-5} M EDTA. This concentration of EDTA binds all traces of free zinc and magnesium ions present in the reagents, but does not yet inhibit the enzyme. A selective partial removal of the zinc or the magnesium could be realized by a dialysis at pH 6 against

so small concentrations of EDTA and *o*-phenanthroline that the metal ions were only very slowly removed from the enzyme. These two chelators show a different affinity for magnesium and zinc ions, like it appears from the corresponding dissociation constants of the metal-chelator complexes at pH 6:
[Mg-EDTA]: $K_1 = 10^{-4.05}$; [Zn-EDTA]: $K_1 = 10^{-11.85}$; [Zn-(*o*-Phen)$_3$]: $K = 10^{-16.6}$; the dissociation constant of Mg-(*o*-Phen)$_x$ is not known.

On the other hand, the dissociation constants of the zinc-phosphatase complex were reported for the *E. coli* enzyme and the bovine kidney alkaline phosphatase. The values indicated in the literature are: $K_1 = 10^{-7.66}$ and $K_2 = 10^{-10}$ to 10^{-11} [9–11]. The dissociation constant of the magnesium-enzyme complex is not exactly known. *Fernley* [12] deduced a value of 5×10^{-4} from the activation curve of the intestinal alkaline phosphatase by magnesium salts. On the basis of the results we obtained for the same enzyme with the method indicated by *Burton* [13] using Eriochrom T as magnesium ligand, we could estimate this constant to be approximately 10^{-6}. A value between these two results was found by *Cathala et al.* [14] for the bovine kidney phosphatase: 5.5×10^{-5}. The selective removal of only the magnesium during the dialysis against a 10^{-4} M EDTA confirms the smaller affinity of this ion for the enzyme compared with that of the zinc ion. The dialysis of the phosphatase against 10^{-4} M *o*-phenanthroline, which is a strong chelator for zinc and a weak one for magnesium, removes in a 10^{-4} M concentration selectively the zinc and lets intact the magnesium content of the enzyme. The simultaneous determination of the metal content and the enzyme activity shows, that the dialysis against *water* removes only the excess of magnesium ions bound to secondary sites without reduction of the specific activity. On the contrary, the removal of the *firmly* bound magnesium reduces the activity to about 40%, even if the zinc content remains intact. We can therefore admit, that the 4 g-atom of tightly bound magnesium are absolutely necessary for the full enzyme activity. On the other hand, a decrease of the zinc content to about 1 g-atom per mol enzyme with a constant magnesium content (4 g-atom per mol) diminishes the phosphatase activity to about 10%. This corroborates the known direct dependence of the activity upon the zinc content. The reduction of the activity to 46%, if the zinc content is reduced to about 3 g-atom proves, that all 4 g-atom of zinc are essential for the activity of the calf intestinal alkaline phosphatase. The exact function of the two metal ions is not known.

In order to determine the influence of the decreased metal content on the V_{max}- and the K_M-values, we measured the enzyme activity with five different substrate concentrations (s. *Exper. Part* and *Table 2*).

The diminution of the K_M-value with the reduced zinc content is not easy to understand, if this metal ion is directly concerned with the formation of the enzyme-substrate complex [15]. It can be easier understood, if the zinc plays a catalytic function (OH-group donor) not directly involved in the substrate fixation, like it is proposed by *Ahlers* [16] for the kidney alkaline phosphatase. The *Lineweaver-Burk* diagram in function of the zinc content *(Fig. 3)* shows, that the increasing lack of zinc in the phosphatase lowers the activity somewhat like the activity change observed during a non-competitive inhibition, but the curves show no common

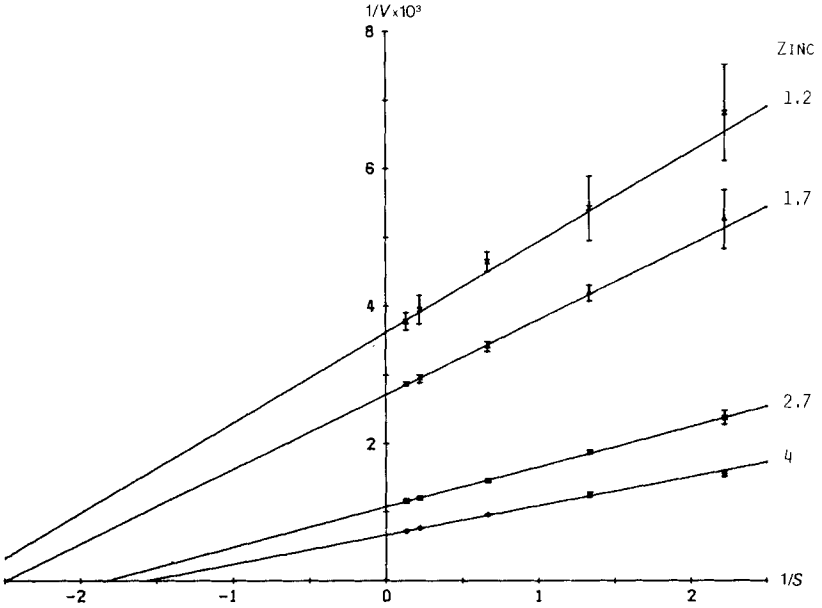

Fig. 3. Lineweaver-Burk *plot of the activity change during the dialysis of the phosphatase against* 10^{-4} M *o-phenanthroline in function of the zinc content* (4, 2.7, 1.7 and 1.2 g-atom per mol). Ordinate: $1/V_{max} \times 10^3 \pm$ S.D.; Abscissa: $1/S$ in mmol/l.

intersection point on the abscissa. The fact that the four curves converge in pairs, corresponding to two different K_M-values, correlates perhaps with a different function of two of the four zinc ions, like it is admitted by *Simpson et al.* [17] for these metal ions in the *E. coli* phosphatase.

The reactivation assays show two anomalies: the reactivation of the partially dezincated phosphatase by magnesium acetate alone and the lack of the reactivation of this phosphatase by zinc acetate, if no magnesium is added. In the first case the reactivation can be explained by the traces of zinc salts present in the magnesium acetate (*Merck 5819;* 0.0002%). The respective molar concentrations after the addition of the magnesium acetate were: magnesium 9.8 mM, phosphatase 2×10^{-11} M and zinc (added with the magnesium acetate) 3.4×10^{-8} M. The traces of zinc present in the purest magnesium acetate available exceed the enzyme molarity by a factor 10^3. The other anomaly is more difficult to explain. We have obtained similar results during the reactivation of the intestinal alkaline phosphatase demetallized and inhibited by high EDTA-concentrations (5×10^{-3} M). The addition of zinc acetate alone did activate the enzyme only from 2 to 29%; then, magnesium acetate was added and the activity increased to 80%. *Hiwada et al.* [8] reported a similar influence of magnesium ions on the EDTA treated pig kidney phosphatase. With zinc chloride alone, they could reactivate the enzyme only to 30%, whereas additional magnesium chloride increased the activity to 100%. These results confirm, that the reintroduction of the zinc ions in the demetallized enzyme is

greatly facilitated in presence of magnesium salts. Moreover, the greater thermostability of the intestinal alkaline phosphatase in presence of magnesium ions indicates that there exists an influence of these ions on the spatial stability of the enzyme.

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Experimental Part

The alkaline phosphatase was isolated from calf intestinal mucosa according to [18]. The *enzyme activity* was determined kinetically at 25° under standard conditions [18] [19], but without addition of magnesium acetate to the substrate-buffer solutions (10 mM *p*-nitrophenylphosphate in 1 M 'diethanolamine' (iminodiethanol) buffer, pH 9.8). To suppress, during the assay, possible enzyme reactivation of the dialyzed preparations by traces of metal ions present in the reagents, 5×10^{-5} mol of EDTA/l was included in the substrate-buffer solutions. V_{max}- and K_M-values were determined with five different substrate concentrations (0.45, 0.75, 1.5, 4.5 and 7.5 mM *p*-nitrophenylphosphate). In this case, the activity was determined with the automatic *Kem-O-Mat TM analyzer (Coulter)*.

The *reactivation* of the dialyzed phosphatase was studied kinetically. To 2 ml of substrate-buffer solution (1 M 'diethanolamine'/HCl pH 9.8; 10 mM *p*-nitrophenylphosphate), 20 µl of diluted enzyme solution were added. The increase of E_{405} was measured during 5 min, then 20 µl of a 1 M magnesium acetate solution or 10 µl of a 1 mM zinc acetate solution or both together were added and the change of E_{405} was measured during 10 min. The reactivation was calculated from the ΔE_{405}/min using an ε_{405}^{mM} for *p*-nitrophenol of 18.5 (*Unicam* spectrophotometer *800*).

Metal determination. The *zinc* and *magnesium content* was determined by atomic absorption photometry *(Zeiss FMD 3)* after addition of 20 µl of HCl-solution (*Merck Suprapur* 30%) and 3 ml of quartz-distilled water to 0.6 ml of the samples removed during the dialysis. Also to the zinc and magnesium standard solutions 20 µl of HCl-solution *Suprapur* were added.

Dialysis. A solution of 18 mg of pure phosphatase (1500 U/mg) in 15 ml of quartz-distilled water (enzyme concentration 10^{-5} mol/l) was dialyzed at 4° in a *Visking* dialysis tubing (1.6 × 10 cm) equipped at the top with a removable stopper, permitting the removal of the samples during the dialysis. The external liquid (100 ml of quartz-distilled water or 100 ml of 10^{-4} M EDTA or 10^{-4} M *o*-phenanthroline) was placed in a 100 ml graduated glass cylinder. The dialysis tubing was dipped so in the external liquid, that the inner and outer meniscus remained always exactly on the same height independently of the inner volume reduction due to the removal of the samples. After a dialysis of 0, 4, 8, 24, 48, 96 and 168 h, a sample of 0.7 ml was removed. Thereof, 0.6 ml were used for the metal determination, the remainder for the enzyme assays. The outer liquid was changed 4 times, after 24, 48, 72 and 96 h. All glassware was cleaned with chromosulfuric acid and rinsed thoroughly with triply quartz-distilled water. The same water quality was also used for the preparation of all solutions needed for the enzyme assays. In order to eliminate the free zinc and magnesium acetate present in the enzyme solution, the phosphatase preparation was precipitated with alcohol (66 vol-%) at −20° just before the dialysis assay, centrifuged and redissolved in quartz-distilled water.

REFERENCES

[1] *L. Engström*, Biochim. Biophys. Acta *52*, 36 (1961).
[2] *M. Fosset, D. Chappelet-Tordo & M. Lazdunski*, Biochemistry *13*, 1783 (1974).
[3] *H.A. Ensinger, H.E. Pauly, G. Pfleiderer & T. Stiefel*, Biochim. Biophys. Acta *527*, 432 (1978).
[4] *E. Sabbioni, F. Girardi & E. Marafante*, Biochemistry *15*, 271 (1976).
[5] *R.T. Simpson & B.L. Vallee*, Biochemistry *7*, 4343 (1968).

[6] *H. Csopak, K. E. Falk & H. Szajn,* Biochim. Biophys. Acta *258,* 466 (1972).
[7] *R. K. Morton,* Biochem. J. *60,* 573 (1955).
[8] *K. Hiwada & E. D. Wachsmuth,* Biochem. J. *141,* 283 (1974).
[9] *S. Cohen & I. B. Wilson,* Biochemistry *5,* 904 (1966).
[10] *H. Csopak,* Europ. J. Biochem. *7,* 186 (1969).
[11] *G. Cathala, Cl. Brunel, D. Chappelet-Tordo & M. Lazdunski,* J. Biol. Chem. *250,* 6040 (1975).
[12] *H. N. Fernley,* 'The Enzymes', P. D. Boyer, ed., Vol. *4,* Academic Press, New York & London 1971, p. 417.
[13] *K. Burton,* Biochem. J. *71,* 388 (1959).
[14] *G. Cathala, Cl. Brunel, D. Chappelet-Tordo & M. Lazdunski,* J. Biol. Chem. *250,* 6046 (1975).
[15] *A. Williams & R. A. Naylor,* J. Chem. Soc. B. *1971,* 1973.
[16] *J. Ahlers,* Biochem. J. *149,* 535 (1975).
[17] *R. T. Simpson & B. L. Vallee,* Biochemistry *7,* 4343 (1968).
[18] *P. Portmann, A. Jörg, K. Furrer, H. S. Walker, P. Leuthard, J. F. Sudan, F. Perriard, J. F. Comment, G. Leva & J. P. Nell,* Helv. Chim. Acta *65,* 2668 (1982).
[19] Empfehlungen der Dtsch. Ges. F. Klin. Chemie, Z. Klin. Chem. Klin. Biochem. *10,* 182 (1972).