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Effects of Zinc and other Metal Ions on the Stability and Activity of *Escherichia coli* Alkaline Phosphatase

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Measurement of the ultraviolet circular dichroism of apo-(alkaline phosphatase) in urea solutions showed substantial denaturation in 3M-urea. A zinc-deficient mutant alkaline phosphatase behaved similarly. The stability of the enzyme in 6M-urea was followed as a function of its zinc content and was found to be dependent on the first two of the four zinc atoms bound by apoenzyme. Phosphatase activity was mostly dependent on a second pair of zinc atoms. Mn^{2+}, Co^{2+}, Cu^{2+} or Cd^{2+} also restored structural stability. Sedimentation-velocity and -equilibrium experiments revealed that dissociation of the dimer accompanied apoenzyme denaturation in urea concentrations of 1M or higher, without treatment with disulphide-reducing agent.

The zinc content of *Escherichia coli* alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) has been determined by many workers, and values reported have generally been 2–4g-atoms of zinc/mol of enzyme. For example, Plocke, Levinthal & Vallee (1962) reported 2g-atoms of zinc/mol; Reynolds & Schlesinger (1967) found 3g-atoms of zinc/mol, and later (Reynolds & Schlesinger, 1968) reported values between 2 and 6g-atoms of zinc/mol. Harris & Coleman (1968) found mean values between 2.5 and 3.5g-atoms of zinc/mol. Simpson, Vallee & Tait (1968) prepared the enzyme with special care for its metal content and reported 4g-atoms of zinc/mol. A number of workers have found the metal-binding capacity of the enzyme to be 4g-atoms of zinc or other metals, for example Lazdunski, Petitclerc & Lazdunski (1969) in studies with Zn^{2+}, Cd^{2+} and Mn^{2+}, Lazdunski *et al.* (1970) with Cu^{2+}, and Simpson & Vallee (1968) with Zn^{2+} and Co^{2+}.

Much recent interest in this enzyme has centred on the possibility of assigning different roles to the two or more separate metal atoms in the dimeric molecule. Successive atoms or pairs of atoms of the same metal appear to bind with different affinity (Simpson & Vallee, 1968); two different zinc dissociation constants have been identified by Cohen & Wilson (1966), although Csopak (1969) found them to be equivalent.

Information about any possible structural, as distinct from activational, role of zinc in this enzyme has to be obtained indirectly, because zinc is diamagnetic and shows no visible transitions. Much of the support for a distinction between roles has been derived from studies of the enzyme in which other metals have been substituted for zinc by reconstitution of the apoenzyme. In the studies described below, zinc-containing alkaline phosphatase was observed in urea denaturant by circular dichroism in the peptide-bond region, and by ultracentrifugation, and its behaviour was related to the bound-metal status. Binding of two zinc atoms was found to control resistance to denaturation fully and a further two zinc atoms governed most of the enzymic activity.

Preliminary communications of this work have appeared (Trotman & Greenwood, 1969, 1971).

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MATERIALS AND METHODS

Chemicals. Urea was Mann Ultra-Pure, supplied by V. A. Howe and Co. Ltd., London W.11, U.K. Dithiothreitol and Chelex 100 were obtained from Calbiochem Ltd., London W.1, U.K. Spectrographically standardized $ZnSO_4$, $CoSO_4$ and $MnSO_4$ were from Johnson Matthey and Co. Ltd., London E.C.1, U.K. Sephadex G-25 and G-100 were purchased from Pharmacia (G.B.) Ltd., London W.13, U.K. *p*-Nitrophenyl phosphate and tris (Trizma base) were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Other reagents were of analytical-reagent quality.

Buffers. Tris buffers were adjusted with HCl to the appropriate pH value by using a glass electrode.

Metal-free solutions. Precautions were taken where required for the exclusion of adventitious metal ions as described by Thiers (1957). Distilled deionized water was stored over Chelex 100 and filtered before use. Solutions

described as metal-free were treated with Chelex 100 and checked with diphenylthiocarbazone (Thiers, 1957).

Preparation of enzymes. Wild-type alkaline phosphatase was extracted from *E. coli* K12, given by Dr W. Hayes. *E. coli* strains $C_{10}F_1$, constitutive for the wild-type enzyme, and $C_{10}F_{18}$, which has a mutationally zinc-deficient inactive phosphatase (Fan, Schlesinger, Torriani, Barrett & Levinthal, 1966), were given by Dr A. Torriani.

Cells were grown in the medium described by Levinthal, Signer & Fetherolf (1962) with DL-threonine, DL-leucine, DL-methionine (each 40mg/l) and thiamin hydrochloride (5mg/l) added for strains $C_{10}F_1$ and $C_{10}F_{18}$, and harvested by continuous centrifugation.

Alkaline phosphatase was extracted and purified by using published techniques (Malamy & Horecker, 1964; Neu & Heppel, 1965) except that a low-molecular-weight impurity absorbing strongly at 260nm was usually present, and was removed by filtration through Sephadex G-100.

Manipulation of metal content of enzyme. Preparation of enzyme containing zinc at intermediate mean values between 0 and 4g-atoms/mol was carried out in two ways; by partial depletion of wild-type enzyme, and by partial reconstitution of apoenzyme. Apoenzyme was prepared by dialysis of 2ml of the wild-type enzyme against 2l of 0.01M-tris–HCl (pH8)–0.01M-EDTA (disodium salt), followed by repeated changes of the metal-free buffer.

Partial zinc depletion of wild-type enzyme was done by reaction in 0.2–0.25ml of the same buffer with 8mM-EDTA (approx. 100-fold molar excess) for various lengths of time from 6min to several hours, followed immediately by separation on Sephadex G-25.

Partial reconstitution was carried out by adding different molar ratios of $ZnSO_4$ to apoenzyme; after 30min the material was passed through a column of Sephadex G-25 to remove unbound or excess of zinc. Uptake of zinc by apoenzyme was equivalent to the amount added, subject to a maximum capacity of almost 4g-atoms/mol if excess of zinc was present.

Experimental measurements. Enzyme concentration was measured spectrophotometrically (Rothman & Byrne, 1963) by taking the molecular weight as 86000 (Schlesinger & Barrett, 1965). Phosphatase activity was assayed by hydrolysis of *p*-nitrophenyl phosphate (0.2mg/ml in 0.1M-tris–HCl buffer, pH8 at 37°C, metal-free). The production of *p*-nitrophenol was followed spectrophotometrically at 410nm and the initial rate determined.

Zinc was assayed by atomic-absorption spectrophotometry in a Unicam SP.90A instrument.

Circular dichroism was measured in a Roussel Jouan Dichrographe CD 185. The optical path was flushed with N_2 when operating below 200nm, and 0.1mm light-path silica sample cuvettes were used. Circular dichroism was expressed as the mean residual molar ellipticity, $[\theta']$, by assuming that the dimer contains 840 amino acid residues [based on Rothman & Byrne (1963) and Schlesinger & Barrett (1965)].

Analytical ultracentrifugation was carried out with a Beckman model E ultracentrifuge with a schlieren optical system. Sedimentation velocity was measured at 59780 or 56100rev./min with the rotor temperature controlled at 20°C. Sedimentation equilibrium was attained after approx. 17h at 9341rev./min, except in experiments with apoenzyme in urea, where the speed was 13410rev./min, including in each case an initial 3h period of overspeeding by 50%. Rotor temperature was measured at equilibrium with the cooling plant set to give a temperature close to 20°C. Molecular weight was computed by Lamm's (1929) method.

RESULTS AND DISCUSSION

Circular-dichroism spectrum of alkaline phosphatase. The spectrum of wild-type enzyme (Fig. 1) has similarities with those of synthetic homopolypeptides and copolymers in the α-helical conformation (Holzwarth & Doty, 1965; Gratzer & Cowburn, 1969) displaying extrema near 222nm, assigned to an oxygen electron $n-\pi^*$ promotion and near 208nm and 191nm, assigned to exciton splitting of the peptide $\pi-\pi^*$ transition in the α-helical conformation. However, the relative ellipticity was less than in model polymers, corresponding to approx. 30% at the $n-\pi^*$ extremum on the basis of recent model-polymer results collated by Gratzer & Cowburn (1969). The same value was obtained when rotational strength was compared with Beychok's (1966) collated model-polymer data.

This spectrum (Fig. 1) forms the basis of subsequent comparisons in the presence of denaturant. The spectrum differs from that presented by Appleby & Coleman (1969) in two regions of interest.

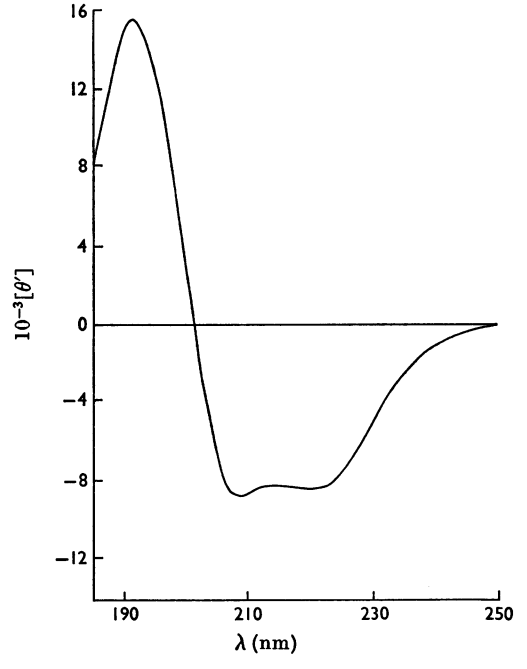

Fig. 1. Circular dichroism of *E. coli* K12 wild-type alkaline phosphatase in the peptide region. Enzyme concentration was 23.6μM in 0.01M-tris–HCl buffer, pH8.

The positive extremum is located at 191nm, compared with their value of 195nm, and has an ellipticity relative to that at 220nm of 1.8, compared with about 1.4. Applebury & Coleman (1969) expressed some surprise that their data could correspond to approx. 60% β-structure, about 30% random coil and only a small amount of α-helix. Reynolds & Schlesinger (1967) obtained a value of 40.5% α-helix from optical-rotatory-dispersion studies. The spectrum in Fig. 1 would appear to lend support to the higher α-helix value, particularly after Jirgensons' (1970) survey of general correlations between circular-dichroic spectra and α-helicity.

Behaviour of alkaline phosphatase in urea solutions. Urea concentrations up to 8M did not significantly change the circular-dichroic spectrum of wild-type enzyme in the accessible part of the peptide region. Dialysis against EDTA did not significantly change the circular-dichroic spectrum in buffer; however, the enzyme then reacted quite differently in urea (Fig. 2). With increasing urea concentration a progressive spectral transformation occurred, with an isodichroic point near 207nm and development of a negative extremum below 203nm. On the basis of polypeptide data (Holzwarth & Doty, 1965; Gratzer & Cowburn, 1969) the spectral change was consistent with random-coil formation. Inactive, mutationally altered enzyme from strain $C_{10}F_{18}$ behaved similarly in urea to wild-type EDTA-treated enzyme (Fig. 3). After partial activation by incubation with zinc by Schlesinger's (1966) method, the mutant enzyme behaved like the wild-type in being resistant to spectral change in urea.

Structural stability and zinc content. Structural stability in 6M-urea as a function of mean zinc content was expressed in terms of diminution of $[\theta']_{222nm}$ (Fig. 4). Preparations containing intermediate mean concentrations between 0 and 4g-atoms of zinc/mol of enzyme were prepared from wild-type enzyme by partial depletion and from apoenzyme by partial reconstitution. Structural stability was found to be dependent on the last two zinc atoms removed by chelation and the first two bound during reconstitution, no hysteresis being detectable between the two pathways.

Enzymic activity and zinc content. The relationship between enzymic activity and zinc content was examined initially for confirmatory purposes. Activity in the partially zinc-reconstituted apoenzyme was examined more closely after difficulty was experienced in reproducing the observation of Simpson & Vallee (1968).

The results in Fig. 5 fall closer to model line A, which represents a dependence of enzymic activity on two less-tightly bound zinc atoms that are bound after those concerned in structural stability. Petitclerc, Lazdunski, Chappelet, Moulin &

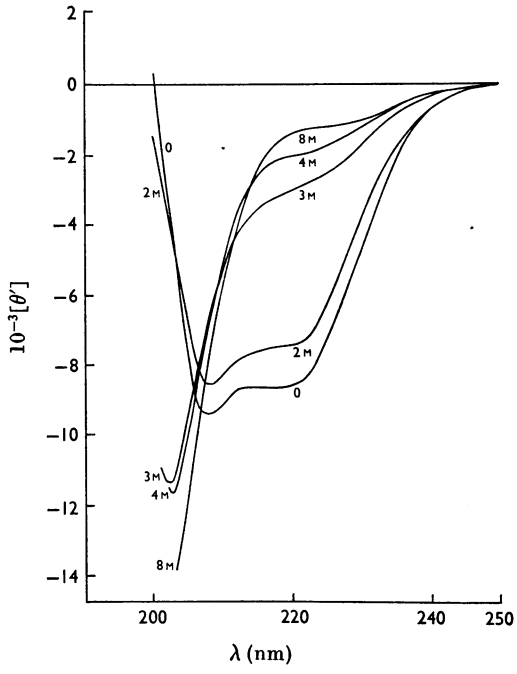

Fig. 2. Circular dichroism of *E. coli* K12 wild-type alkaline phosphatase after dialysis against EDTA, measured in urea of different concentrations. Specimen curves are shown, labelled with the final concentrations of urea.

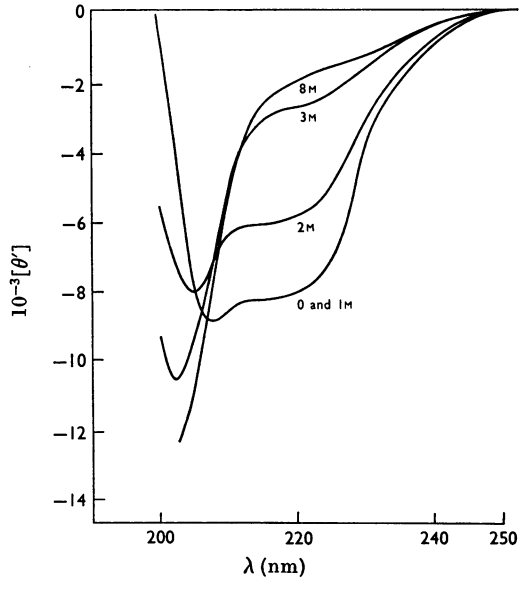

Fig. 3. Circular dichroism of *E. coli* $C_{10}F_{18}$ mutationally zinc-deficient alkaline phosphatase, measured in urea of different concentrations. Specimen curves are shown, labelled with the final concentrations of urea.

Lazdunski (1970) have also suggested that the second pair was implicated in enzyme activation, on the basis of their studies of binding of labelled pyrophosphate. If Fig. 5 is considered alone the deviation of the points from model A could be due to a population of enzyme molecules with different zinc contents (inactive, partially active and fully active), or to partial activation by structural zinc, or to experimental error. The very much lower concentrations of enzyme present during assays of enzyme activity than during circular-dichroism measurements rendered proportionately more important the effect of any adventitious metal ions. However, the results (Figs. 4 and 5), when considered together, are not incompatible with a possible distinction between structural and activational zinc atoms.

The alternative model shown in Fig. 5 (line B) would require restoration of full activity by the two zinc atoms which restored structural stability, but with subsequent binding of a further two functionless zinc atoms. (A number of simplifications are implicit in the straight-line models in Figs. 4 and 5, including the one that dissociation constants are similar within each pair of zinc-binding sites but with a major difference between that of each pair. Each of the two activating zinc atoms would act independently, restoring half the activity).

Replacement by alternative metals. Structural stability in 6M-urea was also fully restored to apoenzyme by a 50-fold molar excess of Mn^{2+}, Co^{2+}, Cu^{2+} or Cd^{2+} (Table 1). Restoration of enzyme activity by Co^{2+} was 9.5%, and by the remaining metals minimal and in accordance with Plocke & Vallee's (1962) studies. A 30-fold excess of Mn^{2+} added to the apoenzyme before a similar amount of Zn^{2+} decreased the reactivation from 100% to 6%, indicating a blocking of activation sites by replacement of Zn^{2+} with Mn^{2+}. Preliminary evidence indicated similar blocking by Cd^{2+}.

Hydrodynamic changes during denaturation. Sedimentation-equilibrium and -velocity determinations on EDTA-treated wild-type enzyme (Table 2) revealed a decrease in molecular weight from 90000 to 40000 on treatment with 6M-urea, together with a decrease in sedimentation coefficient, $s_{20,w}$, from 5.8S to 1.8S and an increase in frictional ratio, f/f_0, from 1.24 to 2.36. These changes were consistent with dissociation of the dimer into monomers accompanied by a high degree of unwinding and exposure to solvent. This dissociation was achieved without a specific disulphide-reducing step. However, subunits prepared from the metal-containing enzyme by the method of Levinthal *et al.* (1962) involving treatment with thioglycollic acid and urea, or by treatment with 10mM-dithiothreitol and urea, also had similar $s_{20,w}$ values (Fig. 6), and metal removal may have been the more significant part of the reaction. Phosphatase inhibition by thioglycollate has been reversed by zinc (Malamy & Horecker, 1964).

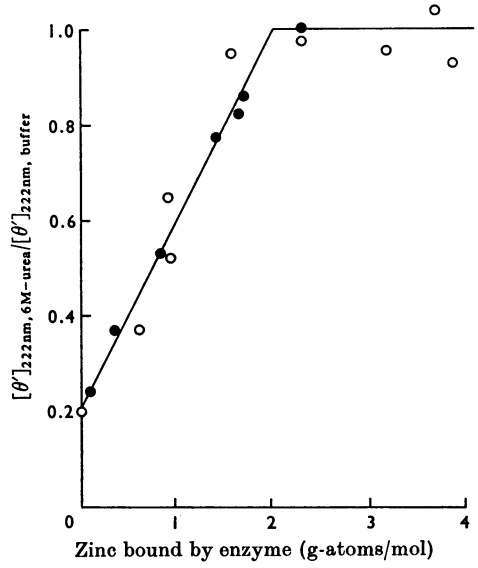

Fig. 4. Mean conformational stability of *E. coli* $C_{10}F_1$ wild-type alkaline phosphatase in 6M-urea as a function of amount of bound zinc. The ordinate shows circular dichroism at 222nm of the enzyme preparation containing 6M-urea, relative to that of the same preparation in buffer only, and the abscissa, the mean bound zinc content of the preparation. ●, Prepared from native enzyme by partial depletion of Zn^{2+}; ○, apoenzyme partially reconstituted by treatment with different concentrations of $ZnSO_4$. The line has been drawn to represent a model in which the molecule is fully stabilized by two zinc atoms but has the capacity to bind four.

Table 1. *Restoration of conformational stability in 6M-urea to* E. coli *$C_{10}F_1$ apo-(alkaline phosphatase) by different metals*

Experimental details are given in the text. Mean conformational stability expresses the retention of native conformation in 6M-urea, in terms of the circular-dichroism band at 222nm (see the text).

Ion	Mean conformational stability $\left(\frac{[\theta']_{222nm,6M\text{-}urea,metal}}{[\theta']_{222nm,6M\text{-}urea,zinc}}\right)$
Mg^{2+}	0.40
Al^{3+}	0.33
Mn^{2+}	1.0
Co^{2+}	1.1
Ni^{2+}	0.58
Cu^{2+}	0.95
Zn^{2+}	1.0
Cd^{2+}	0.98
Nil	0.2

Table 2. *Sedimentation-equilibrium and -velocity determinations on* E. coli $C_{10}F_1$ *wild-type alkaline phosphatase and its apoenzyme before and during treatment with* 6M-*urea*

Values are the means of at least two determinations. Experimental details are given in the text.

	Native enzyme			Apoenzyme		
Medium	Mol.wt.	$s_{20,w}$ (S)	f/f_0	Mol.wt.	$s_{20,w}$ (S)	f/f_0
0.1M-Tris–HCl buffer, pH8	90000	5.8	1.24	90000	5.8	1.24
Buffer+6M-urea	88000	5.4	1.32	40000	1.76	2.36

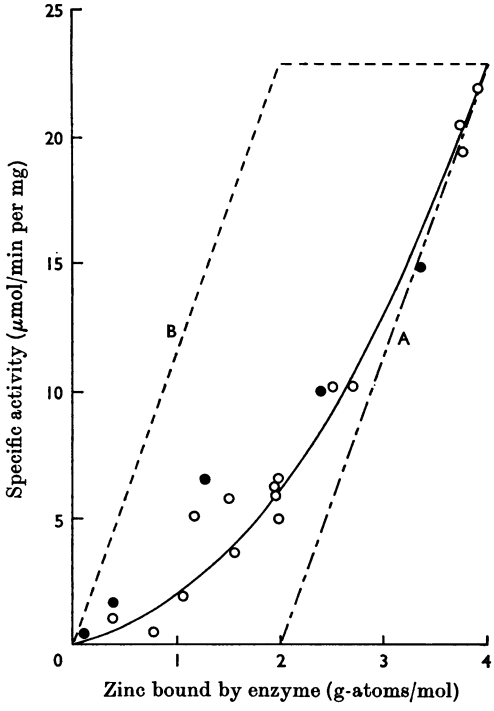

Fig. 5. *E. coli* $C_{10}F_1$ wild-type alkaline phosphatase activity in relation to bound zinc content. ●, Prepared from native enzyme by partial depletion of zinc; ○, apoenzyme partially reconstituted by treatment with different concentrations of $ZnSO_4$. The model lines represent a model dependence of enzymic activity on the third and fourth zinc atoms (A), and on the first and second zinc atoms (B).

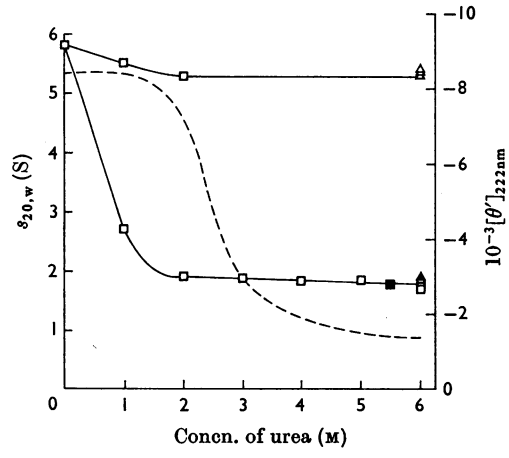

Fig. 6. Behaviour of *E. coli* $C_{10}F_1$ alkaline phosphatase in different concentrations of urea: comparison of sedimentation coefficient ($s_{20,w}$) with conformational stability observed by circular dichroism at 222nm. □, Apoenzyme prepared from wild-type. Two sedimenting species were present in 1M- and 2M-urea; △, wild-type enzyme; ■, subunits prepared from wild-type enzyme by the method of Levinthal *et al.* (1962). ▲, Wild-type enzyme+10mM-dithiothreitol; a trace of dimer also remained. ——, $s_{20,w}$; – – – –, circular dichroism of the apoenzyme at 222nm.

Native enzyme containing 3g-atoms of zinc/mol had $s_{20,w}$ 5.8S, 5% lower than the value established by Reynolds & Schlesinger (1967).

The molecular weight of the wild-type enzyme remained unchanged in urea; changes in $s_{20,w}$ from 5.8S to 5.4S, and in f/f_0 from 1.24 to 1.32, were compatible with a small change in hydration or conformation of the enzyme, and circular-dichroism spectra indicated an essentially unchanged peptide tertiary structure.

Comparison of circular-dichroism with sedimentation-velocity results under conditions of incipient denaturation (Fig. 6) allowed some evaluation of the relative importance of zinc in tertiary and quaternary stabilization respectively. Two sedimenting species were present at concentrations of urea as low as 1M, with approx. 25% of the enzyme as monomer at the enzyme concentration used. This result, in conjunction with the unperturbed peptide circular-dichroic spectrum, suggests dissociation of the enzyme with minimal denaturation. In 2M-urea, approx. 75% of the enzyme had dissociated with an $s_{20,w}$ value almost as low as that reached in 6M-urea, but the circular-dichroic spectrum corresponded with only a small degree of randomization. This might be expected if permeation by solvent and breakdown of

hydrophobic side-chain interactions preceded any substantial loss of secondary structure, as was the case in 3M-urea.

In summary, the enzyme preferentially bound or retained two zinc atoms with complete maintenance of the property of resistance to denaturation in concentrated urea solutions. This high degree of structural involvement is indicative of binding of zinc at specific sites, which may be designated Group 1. Most of the phosphatase activity was dependent on binding of the third and fourth zinc atoms, designated Group 2, and the apparently limiting binding capacity of 4g-atoms argues against non-specific binding. Although this conclusion is compatible with that of Simpson & Vallee (1968) obtained by spectral and activational studies of the cobalt-containing enzyme containing up to four metal atoms, it is converse to their finding in respect of zinc replacement that the first two out of the four g-atoms performed the activational role. However, conclusions similar to ours have been reached on the basis of results from different experiments, referred to above (Petitclerc *et al.* 1970), and the four-site metal-binding capacity has been confirmed with Zn^{2+}, Cd^{2+}, Mn^{2+} and Cu^{2+} (Lazdunski *et al.* 1969, 1970). Although these last authors demonstrated binding of a tightly held pair and of a loosely held pair of Cu^{2+} ions, Csopak & Falk (1970), also using electron paramagnetic resonance, reported different spectral species probably corresponding to one Cu^{2+} and to two Cu^{2+} ions bound, as well as an inability to bind more than approx. 2g-atoms of Cu^{2+}. Applebury & Coleman (1969) also recorded a maximum binding capacity for two g-atoms with Co^{2+}.

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