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Studies on the Interaction of Zinc, Cadmium and Mercuric Ions with Native and Chemically Modified Human Serum Albumin

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The ions of zinc, cadmium and mercury vary widely in their effects on living organisms. Zinc ions are well tolerated in mammals, and distribution studies (Gilbert & Taylor, 1956) have shown that they are located in organs rich in enzymes, such as liver, kidney and testes. Zinc has been found essential to the function of alkaline phosphatase (Mathies, 1958), carboxypeptidase B (Folk, Piez, Carroll & Gladner, 1960), liver alcohol dehydrogenase (Vallee & Hoch, 1957), glutamic dehydrogenase (Adelstein & Vallee, 1958), muscle dehydrogenase (Vallee & Wacker, 1956) and leucocyte carbonic anhydrase (Vallee, Hoch & Hughes, 1954). It is also essential in lower organisms to enzymes such as yeast alcohol dehydrogenase (Vallee & Hoch, 1955) and hexokinase in *Neurospora crassa* (Medina & Nicholas, 1957). The requirement of zinc for these enzymes does not necessarily always imply direct zinc-protein linkage. Nevertheless, if such an interaction is involved, a detailed study of the interaction of Zn^{2+} ions with albumin over a range of pH should provide a model with which to compare the effect of pH on these enzymes.

Cadmium ions, although chemically similar to Zn^{2+} ions, are toxic to many organisms. They have been found, however, in the cortex of the normal horse kidney in a protein also containing Zn^{2+} ions (Kägi & Vallee, 1960).

Mercuric ions are almost universally toxic to living matter. This toxicity is associated with the high affinity for protein thiol groups.

The affinity of proteins for these three metal ions has led to their use as precipitants in plasma-fractionation procedures. Zinc ions were used by

Ketz & Mahl (1955), Cd^{2+} ions by Tolok (1959) and Hg^{2+} ions by Astrup, Schilling, Birch-Anderson & Olsen (1954).

Though previous workers have studied the interactions of these ions with various soluble proteins and have postulated their sites of binding, there are no studies over a range of pH. In this paper quantitative studies are reported on the interactions of native human albumin and some of its chemical modifications with the ions of zinc, cadmium and mercury over the accessible pH range. The techniques used were equilibrium dialysis and radioactive-isotope dilution.

METHODS

Zinc. $^{65}ZnCl_2$ of approximate specific activity 100 mc/g. of Zn was obtained from The Radiochemical Centre, Amersham, Bucks. Carrier inactive $ZnSO_4$ was added to bring the specific activity to the required value.

Cadmium and mercury. Both these materials were obtained by neutron irradiation of inactive material in BEPO at A.E.R.E., Harwell. Cadmium metal (0.5 g.) was irradiated for 4 weeks at a neutron flux of $10^{12}/cm.^2/sec.$ Mercuric oxide (2 g.) was irradiated for 4 weeks at a neutron flux of $1.2 \times 10^{12}/cm.^2/sec.$ No significant quantities of isotopes of other elements are produced by these irradiations. Both samples were dissolved in warm 2N- HNO_3 . Portions were diluted to the required concentration and activity before use.

Measurement of radioactivity. The γ -radiation of each isotope was used for isotope-dilution analysis. Samples (10 ml.) of each solution to be analysed were placed in an annular NaI crystal attached to a photomultiplier and an Ekco automatic scaler. Each sample was counted for a minimum of 160 000 counts.

Equilibrium dialysis. All dialyses were carried out with 10–14 ml. of 0.5–1.0% (w/v) protein solution inside Visking cellulose membranes. The outer solution (120 ml.) was of known pH and I 0.15. For most experiments the concentrations of the metal ions were (mg./l.): Zn^{2+} , 10; Cd^{2+} , 10; Hg^{2+} , 20. Each dialysis was done in a dialysis rocker overnight. Control experiments showed that with this apparatus 0.15M-NaCl soln. came into equilibrium with water in 3 hr.

Outer solutions. For the range pH 4–6 sodium acetate–acetic acid buffers were used. The range pH 6.5–8.5 was covered with either 0.15M-sodium acetate or mixtures of 0.15M- $NaHCO_3$ and 0.15M-NaCl.

Protein concentration. Protein was estimated by semi-micro-Kjeldahl analysis by using a protein:N factor of 6.25.

Albumins. Native human albumin was prepared from plasma by the cold-ether-fractionation technique of Kekwick & Mackay (1954). Chemically modified albumins were prepared by standard procedures as follows: acetylation, Marrack & Orlans (1954); deamination, Fraenkel-Conrat, Bean & Lineweaver (1949); esterification, Chibnall, Mangan & Rees (1958); bromoacetylation, Korman & Clarke (1956); bromopropionylation, Korman & Clarke (1956), β -bromopropionic acid being substituted for bromoacetic acid mole for mole.

Analysis of modified albumins. Amide groups were determined by the method of Chibnall *et al.* (1958) and free amino groups by the method of Van Slyke (1929) as modified by Baddiley, Kekwick & Thain (1952).

Histidine was separated from the protein hydrolysates by the method of Moore & Stein (1951). The separated histidine peak was assayed by the method of Sluyterman (1960). Methoxyl groups were measured by the Zeisel method (Grant, 1951). Pepsin was obtained from The Armour Laboratories as a crystalline material from porcine mucosa.

RESULTS

The effect of pH on the binding of Zn^{2+} and Cd^{2+} ions to albumin is shown in Fig. 1. The pH of maximum binding was pH 7–8 for both ions. No significant quantitative difference was found between bovine and human albumins. Maximum binding for zinc was 8 ± 1 g.atoms/ 10^5 g. of protein, whereas for cadmium it was 4 ± 0.5 g.atoms/ 10^5 g. of protein. Neither ion was bound below pH 4. Mercuric ions reacted differently with albumins, both quantitatively and with respect to pH. Only in the range pH 2.5–4 were the complex systems soluble. At more alkaline pH values the Hg^{2+} ion was precipitated as its hydroxide. In the pH range used the binding was constant at 104 ± 2 g.atoms of mercury/ 10^5 g. of human albumin and 130 ± 2 g.atoms/ 10^5 g. of bovine albumin.

Acetylation of human albumin increased the maximum ion-binding to 9.5 ± 1.0 g.atoms of zinc (Fig. 2) and 6.7 ± 0.8 g.atoms of cadmium/ 10^5 g. of protein. It was not possible to study Hg^{2+} ion-binding as this modified protein was insoluble in the pH range suitable for this ion. Deaminated albumin (Fig. 2) had similar but somewhat decreased ion-binding properties to acetylated albumin. Attempts

Fig. 1. Effect of pH on Zn^{2+} (a) and Cd^{2+} (b) ion-binding to human albumin (●, ▲) and bovine albumin (○, △).

Fig. 2. Effect of pH on the binding of Zn^{2+} ions to acetylated (●), deaminated (△) and esterified (○) human albumins.

to eliminate by esterification the carboxyl groups as binding sites on the native albumin produced a protein with very low affinities for Zn^{2+} (Fig. 2) and Cd^{2+} ions. The maximum values found were 0.7 ± 0.2 g.atom of zinc and 0.8 ± 0.2 g.atom of cadmium bound/ 10^5 g. of protein at pH 7.5.

A derivative with unusual metal-ion-binding properties was prepared by reaction of bromoacetic acid with native albumin. This derivative had 36 ± 3 g.amino groups blocked by the reagent. It

bound a maximum of 81 ± 2 g.atoms of zinc and 78 ± 2 g.atoms of cadmium/ 10^5 g. of protein at pH 6 (Fig. 3). At more alkaline pH the system was unstable and the Zn^{2+} ions were precipitated as the hydroxide. This protein derivative and its mercury complexes were sufficiently soluble to be studied over the range pH 2.9–5.8 (Fig. 3). Maximum Hg^{2+} ion-binding was 190 ± 4 g.atoms/ 10^5 g. of protein. A similar protein derivative with 63 ± 3 substituted amino groups was made with β -bromopropionic acid. This protein still had greater metal-ion-binding properties than the native material but less than the bromoacetic acid derivative (Fig. 3). The maximum binding values observed were 18 g.atoms of zinc at pH 6.2 and 15 g.atoms of cadmium at pH 7.4/ 10^5 g. of protein. At more alkaline pH the metal ions began to be precipitated as their hydroxides. In the accessible range for Hg^{2+} ion-binding (pH 3.4–5.1) a similar effect was observed. The binding value rose from 1 to 7 g.atoms of mercury/ 10^5 g. of protein. The chemical analyses for the modified albumins used are shown in Table 1. A comparison of the variations of Zn^{2+} ion-binding of human albumin and pepsin is shown in Fig. 4.

Fig. 3. Effect of pH on the binding of Zn^{2+} (●), Cd^{2+} (△) and Hg^{2+} (○) ions to (a) bromoacetic acid-treated albumin, and (b) β -bromopropionic acid-treated albumin.

Fig. 4. Comparison of the binding of Zn^{2+} ions to human albumin (○) and porcine pepsin (●) as a function of pH.

Table 1. Chemical analyses of native and chemically modified proteins

For methods of preparation and analysis see text.

Preparation	Moles/ 10^5 g. of protein			
	Amide	Amino	Histidine	Methoxyl
Native human albumin	64 ± 2	87 ± 3	23 ± 1	*
Acetylated albumin	66 ± 2	32 ± 3	23 ± 1	*
Deaminated albumin	62 ± 2	33 ± 3	14 ± 1	*
Esterified albumin	56 ± 2	97 ± 3	19 ± 1	120
Carboxymethylated albumin	*	51 ± 3	3 ± 2	*
β -Carboxyethylated albumin	*	24 ± 3	*	*

* Not determined.

DISCUSSION

The small numbers of Zn^{2+} and Cd^{2+} ions bound per 10^5 g. of albumin suggest that the number of sites available for binding is small. Many authors have concluded that the binding sites were the imidazole residues of histidine (Gurd & Goodman, 1952; Saroff & Mark, 1953; Gurd & Murray, 1954; Rao & Lal, 1958; Vodrazka, 1959; Waldmann-Meyer, 1960). Their evidence was obtained from equilibrium dialysis, polarographic, electrophoresis and chemical modification studies. These authors did not agree about the properties and numbers of sites per molecule. Saroff & Mark (1953) found eight uniform sites whereas Rao & Lal (1958) found two sites, which were different from the remainder in that they bound zinc preferentially to cadmium. Kacena (1954) found that there were 50 or more binding sites for zinc. Waldmann-Meyer (1960) found 17 sites per molecule of pK 7.1. He suggested that the sites of binding were the imidazole residues, because they had the correct pK and were equal in number to the histidine content of albumin. At first sight the results shown in this paper appeared to confirm the imidazole-binding-site hypothesis. The results obtained with acetylated, deaminated and esterified albumin (Table 1) showed, however, that this theory of binding was unsatisfactory. Deliberate chemical modification of the free amino groups of the protein enhanced the metal-binding of both Zn^{2+} and Cd^{2+} ions. The significant effect of acetylation on the protein was to block positively charged groups, so making the protein more electronegative. Deamination had a similar effect but also destroyed 40 % of the histidine residues in the native protein. Zinc and Cd^{2+} ion-binding was similar for these two preparations despite the difference in histidine content. Esterification of the carboxyl groups decreased metal-ion-binding to less than 1 g.ion/ 10^5 g. of albumin. In this preparation the histidine content was not significantly altered by the chemical modification of the protein. If the imidazole residues of the protein alone were responsible for Zn^{2+} and Cd^{2+} ion-binding, then this modification should have shown similar binding to native albumin. The values observed suggest that the single thiol group was responsible in these experiments. Kägi & Vallee (1960) showed that in the cadmium-containing metallothionein the thiol groups were the sites of binding.

To test the validity of the histidine-binding theory equilibrium-dialysis studies with Zn^{2+} ions and pepsin were tried. This protein had six histidine residues/ 10^5 g. of protein (Brand, 1948), compared with 23 for human albumin. The results (Fig. 4) did not show the expected difference between the two proteins. They did show the same relative change as the difference in free carboxyl groups, which number

140 for albumin and 105 for pepsin in each 10^5 g. of protein.

The results with pepsin and native and esterified albumins do not show a specific 1:1 carboxyl to metal-ion-binding. This implies an overall charge effect on the molecule rather than specific sites. Scatchard (1949) expressed the opinion that it was not necessary for the metal ion to react at the site from which the H^+ ion was displaced. He explained this by assuming that it was not the free metal ion which reacted with the protein, but a complex of metal ion and buffer. The combination of the ion with the buffer produced an electrostatic effect similar to the displacement of a H^+ ion. He concluded that buffered systems 'should not be used in the quantitative study of equilibria in protein solution'. The experimental difficulties when excluding buffers other than the protein itself preclude any extensive studies with metal ions. Moreover, in biological systems some additional buffer system is usually present. A further study of metal-protein complexes and the role of different buffer systems is required.

The pH for maximum binding was in the physiological range 7-8 for both Zn^{2+} and Cd^{2+} ions. This suggests that enzymes which need to combine directly with the metal ions in this pH range will be able to make maximum use of the ions available in the system. The present experiments did not show any unusual chemical properties to account for the toxicity of cadmium compared with zinc. But Kägi & Vallee (1960) showed that Cd^{2+} ions were bound in preference to Zn^{2+} ions to the thiol groups of metallothionein. This inversion of the preference for Zn^{2+} ions, shown by those polar groups of proteins other than thiols, suggests that cadmium toxicity is related to the thiol interaction. This reaction relates cadmium to mercury, which is known to have toxic effects due to its combination with thiol groups of proteins.

The species difference of bovine and human albumin with Hg^{2+} ions may be due either to spatial differences of the carboxyl groups or to hydrolysis of some amide groups of bovine albumin in the presence of Hg^{2+} ions. At present there is no evidence to explain these observations. Studies with the complexes of amino acids and Hg^{2+} ions (Perkins, 1952) showed that they had remarkable binding affinities. The high values for the number of ions bound reflected this property. The only numerically possible sites of interaction for Hg^{2+} ions were the carboxyl groups. The known high affinity of the thiol group for Hg^{2+} ions would have accounted for only 1 g.ion/ 10^5 g. of protein.

The present experiments showed that free Hg^{2+} ions would not exist at the pH of most biological systems. However, Saroff & Mark (1953) showed that the predominantly covalent mercuric chloride

combined with albumin at pH 7.5. It is well known that this reagent will react with thiol groups. Both the toxic metals cadmium and mercury thus had a greater affinity than zinc for the thiol groups of proteins. It is therefore probable that the functional groups responsible for the toxic action of the compounds of these two metals are the thiols, although any excess of metal ions will combine with other groups.

Lal & Rao (1957) have shown that Zn^{2+} ions can also combine with albumin as a complex. At pH 6.5 the complex bound was zinc acetate. It is probable that in biological systems in which proteins and metal ions interact, account must be taken of the buffer systems other than protein present. As yet there is no clear evidence to show whether the metal-enzyme systems are examples of the general metal-ion-binding shown by albumin or whether there are unusual prosthetic groups conferring more specific properties.

Treatment of human albumin with bromoacetic acid and β -bromopropionic acid greatly enhanced the ion-binding properties for all three metal ions studied (Fig. 3). The bromoacetic acid-treated protein was the more potent and was also capable of acting over a wider range of pH. Korman & Clarke (1956) showed that bromoacetic acid combined with the amino groups of proteins to produce derivatives of the structure $-\text{N}(\text{CH}_2\text{CO}_2\text{H})_2$. This structure is half of the well-known metal-chelating agent ethylenediaminetetra-acetic acid. Schwarzenbach & Freitag (1951) showed that the log stability constants for the zinc and cadmium complexes of this acid were 16.5 and 16.4 respectively. It seems probable that similar constants can be applied to the modified protein, as the zinc- and cadmium-binding values are little different. The only reactive groups present in proteins with similar constants are the thiols. Kägi & Vallee (1960) obtained values of 17 and 14 for the stability constants of the complexes with Cd^{2+} and Zn^{2+} ions respectively with metallothionein. Both the bromoacetic acid- and bromopropionic acid-modified albumins may be of value in introducing or transporting metal ions in systems where the free metal ions would be precipitated.

SUMMARY

1. Zinc and Cd^{2+} ions combine with native human and bovine albumins on similar or identical sites.

2. At pH 2.5–4.0, 130 g.ions of mercury combine with 10^5 g. of bovine albumin and 100 g.ions with 10^5 g. of human albumin.

3. Evidence is produced to suggest that the binding of Zn^{2+} and Cd^{2+} ions to native albumin is governed by the electronegative charge on the molecule.

4. The metal-ion-binding properties of albumins treated with bromoacetic acid and bromopropionic acid are reported.

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