Studies on the Interaction of Magnesium, Calcium and Strontium Ions with Native and Chemically Modified Human Serum Albumin

By L. I. IRONS AND D. J. PERKINS

Chemical Pathology Department, St George's Hospital Medical School, London, S.W. 1

(Received 31 October 1961)

The interactions of Ca^{2+} ions with plasma proteins were first studied by Pribram (1871). Since then there have been limited studies with Mg^{2+} , Sr^{2+} and Ba^{2+} ions. The biological interest in the alkaline-earth cations arises from their part in bone formation and the hazards of radioactive fallout. Carr & Woods (1955) compared the binding properties of bovine albumin with all four ions at a single pH and in the absence of sodium chloride; they found that the ions behaved in an identical fashion. Similar results were obtained by Van Os & Koopman-van Eupen (1957) in a study of Ca^{2+} ion- and Mg^{2+} ion-binding to human albumin. These results do not parallel the binding affinities shown by these ions towards small ligand molecules, where the order generally found is:

Mg^{2+} , $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}$ ions,

although Mg^{2+} ion is often irregular in position (Schubert, 1954).

In this paper an attempt has been made to extend this work by comparing the binding of the alkaline-earth ions to native and chemically modified human albumin over a range of pH and in the presence of near-physiological concentrations of Na^+ ions. Additional information about the nature of the binding sites on albumin for these ions is given. Greenwald (1926) postulated the existence of a citrate-like substance in blood capable of holding calcium in a non-ionized form. Several workers in the period 1926–30 studied the behaviour of Ca^{2+} ions in the presence of citrate but no information is available about its effect on the Ca^{2+} ion-binding to purified proteins. Since hydroxy acids are present in biological systems, information of this kind is necessary for an understanding of the role played by Ca^{2+} ions in living organisms. The effect of citrate ions on the Ca^{2+} ion-binding of native albumin was also investigated.

EXPERIMENTAL

Radioactive solutions. Carrier-free chloride solutions of ^{45}Ca , ^{89}Sr and ^{28}Mg were obtained from The Radiochemical Centre, Amersham, Bucks. The isotopes were diluted before use with inactive carrier to give solutions of known metal-ion concentration and activity. The short half-life of ^{28}Mg limited the number of experiments carried out with this material.

Measurement of radioactivity. The method of isotope dilution was used for the quantitative determination of metal ions. The isotope ^{28}Mg was counted with a well-crystal system in conjunction with an automatic scaler.

The isotopes ^{45}Ca and ^{89}Sr were counted by a β -particle liquid-scintillation technique at -17° . Diphenyloxazole (0.3%, w/v) in toluene was used as the organic scintillator. Aqueous samples (1 ml.) were blended with a scintillator (5 ml.) with ethanol (10 ml.). Protein solutions were precipitated by the ethanol in the counting solution. When the precipitated protein was allowed to settle in the counting dish it did not appreciably affect the count. In practice, therefore, the protein was precipitated and kept for at least 3–4 hr. in the dish at -17° before counting. Solutions containing buffer ions in the presence and absence of protein were counted with the same efficiency as an aqueous solution containing the same amount of activity. Each sample was counted for a minimum of 150 000 counts.

Equilibrium dialysis. All dialyses were carried out with 10 ml. of 0.5–0.7% (w/v) protein–buffer solution inside Visking cellulose membranes. The outer solution (120–140 ml.) was of measured pH and molarity 0.15–0.17. Each dialysis was done in a dialysis rocker overnight at room temperature. Control experiments showed that with this apparatus 0.10M-sodium chloride came into equilibrium with water in 3 hr. Dialysis experiments as a function of pH were carried out at fixed Ca^{2+} , Mg^{2+} and Sr^{2+} ion concentrations of 20, 10 and 40 mg./l. respectively.

Outer buffer solutions. The following buffer systems were used: pH 2–4, 0.05M-potassium hydrogen phthalate; pH 4–6, 0.05M-sodium acetate–acetic acid; pH 6–7.5, 0.02M-sodium cacodylate; pH 8–9.5, 0.1M-sodium hydroxide–boric acid–potassium chloride. Sodium chloride was added to each buffer solution to bring the molarity to 0.15–0.17.

Protein concentration. Protein was estimated by a semi-micro-Kjeldahl technique; a protein:N factor of 6.25 was assumed.

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: esterification, by the method of Chibnall, Mangan & Rees (1958); acetylation, by the method of Marrack & Orlans (1954); bromoacetylation, by the method of Korman & Clarke (1956); phosphorylation, by the method of Ferrel, Olcott & Fraenkel-Conrat (1948).

Analysis of modified albumins. Amide groups were determined by the method of Chibnall *et al.* (1958), and free amino groups by the method of Baddiley, Kekwick & Thain (1952). Methoxyl groups were measured by the Zeissel method (Grant, 1951) and phosphorus by the method of Allen (1940) after correction for inorganic orthophosphate (Lowry & Lopez, 1946).

Table 1. *Chemical analysis of native and chemically modified proteins*

For methods of preparation and analysis, see text. —, Not determined.

Preparation	Moles/10 ⁵ g. of protein			
	Amino	Amide	Methoxyl	Phosphorus
Native human albumin	100	64	—	—
Esterified albumin	103	54	76	—
Acetylated albumin (1)	50	59	—	—
Acetylated albumin (2)	47	66	—	—
Phosphorylated albumin (1)	100	63	—	64
Phosphorylated albumin (2)	98	60	—	73
Bromoacetylated albumin	37	74	—	—

Fig. 1. Effect of pH on the binding of Ca²⁺ (○), Mg²⁺ (△) and Sr²⁺ (●) ions to human albumin. Fig. 2. Effect of pH on the binding of Ca²⁺ (○) and Sr²⁺ (●) ions to acetylated albumin (1) and of Ca²⁺ ions to esterified albumin (△).

RESULTS

The chemical analyses of the modified albumins used are shown in Table 1. Acetylated albumins (1) and (2) refer to two different preparations from the same batch of native albumin. The errors of measurement are (groups/10⁵ g. of protein): amino, ± 3 ; amide, ± 2 ; methoxyl, ± 4 ; phosphorus, ± 3 .

Table 2. *Binding of magnesium ions to acetylated and phosphorylated albumins*

For experimental details, see text.

pH	Mg ²⁺ ions (moles/10 ⁵ g. of protein)	
	Acetylated albumin (2)	Phosphorylated albumin (2)
5.5	0.4	1.6
5.8	0.7	2.5
6.1	1.0	2.3
6.4	0.5	3.6
7.0	1.0	4.6
7.8	0.7	6.6
8.6	1.2	7.7
9.0	1.9	8.3

Fig. 1 shows the effect of pH on the binding of Ca²⁺, Mg²⁺ and Sr²⁺ ions to native albumin. Binding starts at about pH 5 and increases with pH. The relative binding sequence was:

Ca²⁺ > Mg²⁺ > Sr²⁺ ions.

The binding curves show that net charge is important. Up to pH 6 carboxyl groups may be the sites of binding. At this pH all these groups are ionized. The increase in binding at more alkaline pH can be explained by electrostatic attraction as the protein molecule becomes more negatively charged. The possibility of binding through imidazole groups above pH 6 is unlikely because alkaline-earth ions have little affinity for these groups (Orgel, 1958).

Fig. 2 shows the effect of pH on the binding of Ca²⁺ and Sr²⁺ ions to acetylated and esterified albumins. Binding of Ca²⁺ ions to acetylated albumin (1) starts at about pH 5, increases with increasing pH and reaches a plateau of 2.4 ± 0.2 moles bound/10⁵ g. of protein between pH 7.5 and 9.0. Binding of Sr²⁺ ions to this protein increases with increasing pH but shows no plateau in the alkaline region and is less than the binding of Ca²⁺ ions. Results of Mg²⁺ ion-binding to acetylated albumin (2) are shown in Table 2: they give a binding curve very similar to that of Sr²⁺ ions. Binding of Ca²⁺ ions to esterified albumin starts at pH 6.7, increases with increasing pH and reaches

Fig. 3. Effect of pH on the binding of Ca^{2+} ions (○) to phosphorylated albumin (1), Sr^{2+} ions (●) to phosphorylated albumin (2), and of Ca^{2+} (Δ) and Sr^{2+} (▲) ions to bromoacetylated albumin.

Fig. 4. Effect of increasing free metal-ion concentration on the binding of Ca^{2+} ions (Δ) at pH 7.50 and of Ca^{2+} (○) and Sr^{2+} (●) ions at pH 6.50 to human albumin.

Fig. 5. Effect of pH on the binding of Ca^{2+} ions to native (○) and esterified (Δ) human albumin in the presence of sodium citrate and to human albumin (●) in the absence of sodium citrate.

a maximum of 0.8 ± 0.1 mole bound/ 10^5 g. of protein at pH 7.5. At higher pH values, low results are obtained owing to precipitation. Binding of Sr^{2+} and Mg^{2+} ions to esterified albumin is less than 0.1 mole/ 10^5 g. of protein over the pH range 5.0–7.5. Comparison of Figs. 1 and 2 shows that acetylated albumin binds more and esterified albumin fewer metal ions than native albumin.

The effect of pH on the binding of Ca^{2+} and Sr^{2+} ions to phosphorylated and bromoacetylated albumins is shown in Fig. 3. Binding of Ca^{2+} ions to phosphorylated albumin (1) increases from 6 ± 0.5 to 19 ± 1 moles/ 10^5 g. of protein as the pH is raised from 5.0 to 8.5. Binding of Ca^{2+} ions to bromoacetylated albumin is constant at 7.0 ± 0.5 moles/ 10^5 g. of protein up to pH 7.5 and then increases rapidly with increasing pH. Binding of Sr^{2+} ions to these albumins increases with increasing pH but not to the same extent as for Ca^{2+} ions. Results of Mg^{2+} ion-binding to phosphorylated albumin (2) are shown in Table 2 and give a binding curve very similar to that of Sr^{2+} ions. Comparison of Figs. 1 and 3 shows that phosphorylated and bromoacetylated albumins bind more metal ions than native albumin. The relative binding sequence for the modified proteins is: $\text{Ca}^{2+} > \text{Mg}^{2+}, \text{Sr}^{2+}$ ions. The difference between Mg^{2+} ion- and Sr^{2+} ion-binding to these proteins is small.

Fig. 4 shows the effect of increasing free metal-ion concentration on the binding of Ca^{2+} ions to native albumin at pH 6.50 and 7.50 and on the binding of Sr^{2+} ions to native albumin at pH 6.50. Binding increases with increasing metal-ion concentration and takes place in two stages corresponding to two sections of the binding curves.

Fig. 5 shows the effect of pH on the binding of Ca^{2+} ions to native and esterified albumin in the presence of sodium citrate at a citrate ion: Ca^{2+} ion ratio of 5:1. For comparison, the curve for native albumin, as drawn in Fig. 1, is included in this Figure. In the presence of sodium citrate, Ca^{2+} ion-binding to native albumin starts at pH 2.8, reaches a maximum at pH 3.6–3.8, decreases to a minimum at pH 5.2–5.4 and then increases with increasing pH. Binding to esterified albumin is greater than to native albumin and starts at pH 2.8, reaches a maximum at pH 5.4 and then decreases with increasing pH.

Experiments with Ba^{2+} ions were also carried out, but binding occurred between the metal ions and the dialysis membrane. This made results difficult to interpret and they are not reported.

DISCUSSION

The small number of Ca^{2+} , Mg^{2+} and Sr^{2+} ions bound/ 10^5 g. of native albumin suggests that the total number of available sites is small. The

effect of pH on the binding of Ca^{2+} ions to bovine albumin has been reported by Edsall, Edelhoch, Lontie & Morrison (1950), Carr (1953*a*) and Katz & Klotz (1953). Carr (1953*b*) showed that human and bovine albumin have equal binding capacities for Ca^{2+} ions. Carr & Woods (1955), Kepes (1954) and Van Os & Koopman-van Eupen (1957) detected no difference in the binding of the alkaline-earth ions to human and bovine albumin. This does not agree with the results presented here, but the above authors presented only limited experimental results.

Binding of Ca^{2+} , Sr^{2+} and Mg^{2+} ions to native albumin at the isoelectric point is probably zero, which agrees with the Ca^{2+} ion results of Carr (1953*a*) and Katz & Klotz (1953). However, Edsall *et al.* (1950) and Doremus & Johnson (1958) found, from light-scattering and conductance measurements respectively, at a single pH, that Ca^{2+} ions were bound to bovine albumin. Since both these groups of authors made simplifying assumptions in the equations used to calculate the binding, the direct measurements presented here are probably more reliable. It is difficult to make a quantitative comparison of the pH-binding curves since various systems have been used to study the binding. Thus Carr (1953*a, b*) used unbuffered solutions containing Ca^{2+} ions as the only cation, and Katz & Klotz (1953) used bicarbonate buffer solutions containing about 0.1M-sodium chloride.

The effect of increasing Ca^{2+} ion and Sr^{2+} ion concentration on the binding to native albumin gives binding curves different from those reported by Carr & Woods (1955), Van Os & Koopman-van Eupen (1957) and Doremus & Johnson (1958). These authors found binding to increase with metal-ion concentration until a plateau was reached at a free metal-ion concentration above about 12 mM. However, the systems studied contained little or no Na^+ ion, so the results must be characteristic of Ca^{2+} ions in the absence of competing ions and cannot be extended to biological systems containing Na^+ ions.

Although the binding is higher at pH 7.5 than at 6.5 for Ca^{2+} ions, the albumin molecule has the same maximum number of binding sites available at both pH values. This suggests that binding to native albumin occurs through specific sites and also that probably both electrostatic and specific site-binding occur simultaneously. Electrostatic binding is probably responsible for the steep section of the binding-concentration curve, where presumably all the specific sites have been saturated with metal ions.

The importance of net charge on the binding can also explain the results obtained with acetylated and esterified albumin. Acetylation makes the

protein more electronegative and hence increases its capacity to bind metal ions as compared with native albumin. Abels (1936) found egg albumin to have a decreased Ca^{2+} ion-binding capacity after acetylation. This difference may be explained by the different acetylating systems used: Abels used acetic acid-acetic anhydride in quinoline (Freudenberg & Eyer, 1932), whereas we used acetic anhydride in aqueous solution. Esterification makes the protein more electropositive and hence decreases its binding capacity. Binding above pH 6.7 to this protein probably occurs because of a change from net positive to net negative charge near this pH.

The net-charge theory does not, however, explain the high binding obtained with phosphorylated and bromoacetylated albumins. Phosphate groups have a high affinity for alkaline-earth ions so that binding probably occurs specifically through these groups. Osterberg (1957) has shown the phosphate group in serine *O*-phosphate to have pK 5.65 at 25° so that the increase in binding with pH may be a combination of electrostatic effects and specific binding to dissociating phosphate groups. The effect of treating proteins with bromoacetic acid is to convert amino groups into $-\text{N}(\text{CH}_2\cdot\text{CO}_2\text{H})_2$ groups (Korman & Clarke, 1956). This structure is half of the well-known metal-chelating agent EDTA, which binds the alkaline-earth ions strongly. Hence the binding of Ca^{2+} , Mg^{2+} and Sr^{2+} ions to bromoacetylated albumin may occur directly through $-\text{N}(\text{CH}_2\cdot\text{CO}_2\text{H})_2$ groups.

Comparison of the binding curves of Ca^{2+} ions with human albumin in the presence and absence of citrate ions shows that considerable differences in binding occur at pH values below 5. The pK values of the carboxyl groups of citric acid are 2.13, 4.76 and 6.40 (Bates & Pinching, 1949), so that at pH values below 5 the species positively charged calcium dihydrogen citrate, calcium hydrogen citrate and negatively charged calcium citrate are all present at appreciable concentrations. Below pH 5 the protein has a net positive charge and would be expected to bind the negatively charged calcium citrate complex. The minimum in binding at pH 5.2, which is near the isoelectric point of albumin, can be explained by a decreased electrostatic effect as the protein acquires a net negative charge. The increase in binding from pH 6 suggests that Ca^{2+} ions, derived from the dissociation of the negatively charged calcium citrate complex, are bound as the free ions. The binding in this case is lower than that obtained in the absence of citrate ions since calcium citrate is a weak electrolyte and incompletely dissociated. The increased binding to esterified albumin can be explained by increased electrostatic attraction of the negatively charged calcium citrate to the positively charged protein.

The pH at which albumin binds a maximum amount of negatively charged calcium citrate is also the pH at which the molecule undergoes a reversible expansion (Foster, 1960).

Coddington & Perkins (1960) found similar results in their study of the binding of Fe^{3+} ions to human albumin in the presence of citrate. This indicates that in the compact molecule the localization of regions of charge is responsible for the binding of the charged citrate complex. Below pH 3.6–3.8 such regions of charge may be fewer in number, since the forces maintaining a compact structure would be lost and regions of like charge would be able to repel each other. This would be shown by a decreased binding of the expanded molecule for the citrate complex. However, since at acid pH the formation of negatively charged calcium citrate would vary with pH, the shape of the binding curve would be the result of simultaneous formation of the complex and the formation of favourable regions of charge on the molecule capable of binding this complex.

The sequence: $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+}$ ions, found here for the combination of these metal ions with proteins, differs with results obtained by other workers but agrees with results obtained with small ligand molecules. The order generally found for them is: Mg^{2+} , $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}$ ions, although Mg^{2+} ion is often irregular in position (Schubert, 1954; Williams, 1953). It is known from studies with amino acids and peptides that the metal ion predominates in determining the stability constants of the complexes formed. However, it is not possible at present to determine the exact extent to which factors such as ionic strength and molecular charge and size, hydration etc. contribute to the stability of such complexes.

SUMMARY

1. Interactions of Ca^{2+} , Mg^{2+} and Sr^{2+} ions with native and chemically modified human albumin are similar.

2. The relative binding affinity to native albumin is: $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{Sr}^{2+}$ ions, and to the modified albumin $\text{Ca}^{2+} > \text{Mg}^{2+}$, Sr^{2+} ions.

3. The binding to native, esterified and acetylated albumins is governed mainly by electrostatic attraction to non-specific sites, although with native albumin specific site binding has also been found. Binding to phosphorylated and bromoacetylated albumins occurs through specific sites.

4. In the presence of citrate ions, Ca^{2+} ions are bound to native albumin at pH values below 5.2

as a negatively charged calcium citrate complex and at pH values above 5.2 as the free ion.

We wish to thank Professor N. H. Martin for his encouragement during this work and the Medical Research Council for a grant. We are also indebted to the Governors of St George's Hospital, London, S.W. 1, for the provision of apparatus and facilities.

REFERENCES

- Abels, J. C. (1936). *J. Amer. chem. Soc.* **58**, 2609.
 Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
 Baddiley, J., Kekwick, R. A. & Thain, E. M. (1952). *Nature, Lond.*, **170**, 968.
 Bates, R. G. & Pinching, G. D. (1949). *J. Amer. chem. Soc.* **71**, 1274.
 Carr, C. W. (1953a). *Arch. Biochem. Biophys.* **43**, 147.
 Carr, C. W. (1953b). *Arch. Biochem. Biophys.* **46**, 424.
 Carr, C. W. & Woods, K. R. (1955). *Arch. Biochem. Biophys.* **55**, 1.
 Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958). *Biochem. J.* **68**, 111.
 Coddington, A. & Perkins, D. J. (1960). *Biochim. biophys. Acta*, **44**, 361.
 Doremus, R. H. & Johnson, P. (1958). *J. phys. Chem.* **62**, 203.
 Edsall, J. T., Edelhoch, H., Lontie, R. & Morrison, P. R. (1950). *J. Amer. chem. Soc.* **72**, 4641.
 Ferrel, R. E., Olcott, H. S. & Fraenkel-Conrat, H. (1948). *J. Amer. chem. Soc.* **70**, 2101.
 Foster, J. S. (1960). In *Plasma Proteins*, vol. 1, p. 206. Ed. by Putnam, F. W. New York: Academic Press Inc.
 Freudenberg, K. & Eyer, H. (1932). *Hoppe-Seyl. Z.* **213**, 226.
 Grant, J. (1951). In *Pregl's Quantitative Organic Micro-Analysis*, 5th ed., p. 182. London: J. and A. Churchill Ltd.
 Greenwald, I. (1926). *J. biol. Chem.* **67**, 1.
 Katz, S. & Klotz, I. M. (1953). *Arch. Biochem. Biophys.* **44**, 351.
 Kekwick, R. A. & Mackay, M. E. (1954). *Spec. Rep. Ser. med. Res. Coun., Lond.*, no. 286.
 Kepes, A. (1954). *C.R. Acad. Sci., Paris*, **239**, 117.
 Korman, S. & Clarke, H. T. (1956). *J. biol. Chem.* **221**, 133.
 Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.
 Marrack, J. R. & Orlans, E. S. (1954). *Brit. J. exp. Path.* **35**, 389.
 Orgel, L. E. (1958). In *Metals and Enzyme Activity*, p. 8. Ed. by Crook, E. M. Cambridge University Press.
 Osterberg, R. (1957). *Nature, Lond.*, **179**, 476.
 Pribram, R. (1871). *Ber. sächs. Ges. (Akad.) Wiss.* **23**, 279.
 Schubert, J. (1954). In *Chemical Specificity in Biological Interactions*, p. 136. Ed. by Gurd, F. R. N. New York: Academic Press Inc.
 Van Os, G. A. J. & Koopman-van Eupen, J. H. M. (1957). *Rec. Trav. chim. Pays-Bas*, **76**, 390.
 Williams, R. J. P. (1953). *Biol. Rev.* **28**, 390.