A STUDY OF THE INTERACTION OF METAL IONS WITH BOVINE SERUM ALBUMIN

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C_O_N_T_E_N_T_S

GENERAL INTRODUCTION

Proteins interact with a variety of substances, such as organic and inorganic ions, drugs, proteins and the like. Of such interaction products, metallo-proteins and metallo-enzymes form an important group; they occur in biological systems and play a vital role in metabolic processes.

The metallo-proteins such as hemocyanin (copper), hemoglobin (iron), and chlorophyll (magnesium) have been studied in great detail over a number of years. The metal forms an integral part of the protein, and can be removed only by drastic chemical action. The precise role of the metal ion in the physiological function of these proteins has also been fairly well established.

The metallo-enzymes, on the other hand, contain the metal ion in a loosely bound form. The metal can be removed by mild processes such as dialysis. Furthermore, the activation may be brought about by other metals having similar electron configurations. Arginase, for instance, may be activated by Fe^{++}, Co^{++} and Ni^{++} besides Mn^{++}, which is the natural activator (1). In some cases, however, the activation may be brought about by structurally dissimilar metals. It seems that neither the catalytic activity nor the metal requirement of enzyme systems can be easily explained in terms of the nature and electronic configuration of the metal ion alone. The nature of the functional groups and their juxtaposition in the protein molecule may possibly have to be

taken into account. It is precisely for this reason that more and more attention has been paid, in recent years, to a study of interactions in simple systems, involving homogeneous proteins and their constituents in which the nature of the ligand group is fairly well established. Such studies have assumed a further importance in view of the recent use of divalent metal ions, notably zinc ion, in selectively rendering insoluble the proteins contained in human blood plasma[2], and in the extracts of bovine liver[3]. Of the homogeneous proteins, crystalline bovine and human serum albumins have been studied the most, especially, because these proteins are well characterized in terms of their hydrogen ion equilibria[4,5] and because their derivatives can be prepared relatively easily[6,7] ;

(I)

Electron Configuration and Complex Formation

We may, to start with, examine the complex forming ability of a metal ion in so far as it can be deduced from its electron configuration. The ions of the non-transition metals (such as the alkali and alkaline earth metals), in which the inner electron orbits are filled, assume the stable configuration of the nearest noble gas; hence they react with the functional groups by virtue of a net electrostatic attraction. The transition elements, however, frequently utilize their inner electron orbits for covalent bonding and generally form far stronger complexes than the non-transition elements. An

important feature of the transition metal ions is the hybridisation of their d, s and p orbitals, leading to better orbitals for covalent bond formation at specially favoured angles. The electron configuration and the favoured orientation of covalent bonds are given in Table I for a few metal ions. It may be noticed that the 3d orbitals are made available by forcing the unpaired 3d electrons to pair (Mn^{++}, Fe^{++}, Ni^{++}), or by forcing a lone electron from the 3d to 4d orbital (Cu^{++}) or both (Co^{++}). There is thus a magnetic criterion for the formation of covalent bonds involving the transition elements.

Table - I

Metal ion	Coordination number	Electron Configuration			orbitals available for covalent bond formation				Type	
		3d	4s	4p	3d	4s	4p	4d		
Mn^{2}	6	argon core	⊙⊙⊙⊙⊙	○	○○○	⊙⊙⊙	[○○	○	○○○]	d^2sp^3 octahedral
Fe^{2}	6	"	⊙⊙⊙⊙⊙	○	○○○	⊙⊙⊙	[○○	○	○○○]	d^2sp^3 "
Co^{2}	6	"	⊙⊙⊙⊙⊙	○	○○○	⊙⊙⊙	[○○	○	○○○] ⊙	d^2sp^3 "
Ni^{2}	4	"	⊙⊙⊙⊙⊙	○	○○○	⊙⊙⊙⊙	[○	○	○○] ○	dsp^2 square
Cu^{2}	4	"	⊙⊙⊙⊙⊙	○	○○○	⊙⊙⊙⊙	[○	○	○○] ○ ⊙	dsp^2 "
Zn^{2}	4	"	⊙⊙⊙⊙⊙	○	○○○	⊙⊙⊙⊙⊙	[○	○○○]		sp^3 tetrahedral

A real bond between two atoms is, however, seldom entirely covalent, and may be treated as a resonance hybrid between an idealized covalent bond and an idealized ionic bond.

The partial ionic character of a real bond may be related to the electronegativity difference between the interacting atoms. Examples of two electronic patterns for ferric complexes, FeA_6^{---}, with ligand A^- are shown in Table II. If the ferric ion forms complexes without the participation of 3d electrons, as with FeF_6^{---}, the complex will exhibit the electron distribution shown in the upper part of Table II. If, however, the electronegativity of the ligand is sufficiently low, as with $Fe(CN)_6^{---}$, the whole complex can be stabilized by d^2sp^3 hybrid bonding as shown in the lower part of Table II. The quantum-mechanical treatment, developed by Pauling[8], has formed a satisfactory basis for elucidating the nature of complex formation between metal ions and simple ligands.

Table - II

Electron Distributions

Complex	3d	4s	4p	4d
FeA_6^{---}		Ä	Ä Ä Ä Ä Ä	
			ionic octahedral	
	θ θ θ Ä Ä	Ä	Ä Ä Ä	
			d^2sp^3 octahedral	

(II)

Reversible Equilibria and Affinity Constants.

It is conventional to consider a metal ion, in the

valence state in question, acting as a recipient, through its vacant orbitals, of electron pairs from a ligand. A ligand should, therefore, be in a position to donate such electron pairs. Take, for example, the equilibrium

$$- NH_3^+ \rightleftharpoons - \ddot{N}H_2 + H^+ \quad \ldots\ldots\ldots \quad (1)$$

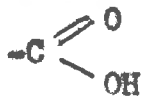
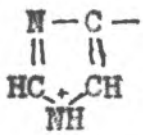
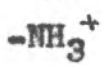
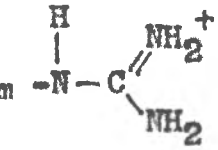
It may be noticed that it is only the basic or the dissociated form, $- NH_2$, which can act as a ligand for bonding with metal ions, the equilibrium conditions being represented as

$$M + :\dot{N}H_2 \rightleftharpoons M:\dot{N}H_2 \quad \ldots\ldots\ldots \quad (2)$$

If there were only one ligand on a protein molecule, the association or the stability constant, k, would represent the complexing ability of the metal ion with the protein. A protein, however, is a composite of several amino acids joined together in a peptide chain. There may thus be a variety of functional groups with varying proton affinities available on a protein molecule. The number of various polar side chains and their pK^o values are given in Table III for human and bovine serum albumins[9,10]. It may be noticed that the carooxyl, imidazole, and amino groups will be expected to oe the reactive sites at pH values around 4, 6 and 8-10 respectively.

Table - III

Polar side chains in Serum Albumin Molecule[9,10].

Group	Structure	Amino acid residue	number present per mole: Human Serum Albumin — Analysis	Human Serum Albumin — Titration	Bovine Serum Albumin — Analysis	Bovine Serum Albumin — Expected Titration	pK^{o}(5) (Tanford)
Free Carboxyl		Aspartic acid & Glutamic acid	101	106	101	106	4.0
imidazole		Histidine	16	16	18	18	6.1
∝-amino			9	4	10	5	8.0
∈-amino		Lysine	58	56	60	58	9.4
Phenolic OH		Tyrosine	18	18	19	19	9.6
Sulfhydral		Cysteine	0.7	-	0.7	-	10
Guanidinum		Arginine	24	24	23	23	12
Total cationic groups			107	100	111	104	
Total cationic less guanidinium			33	76	88	81	

Assuming that a single set of n equivalent sites are responsible

for binding metal ions under given conditions, we may write the successive equilibria as follows :

$$\left.\begin{aligned} P + A &\rightleftharpoons PA \\ PA + A &\rightleftharpoons PA_2 \\ PA_{i-1} + A &\rightleftharpoons PA_i \\ PA_{n-1} + A &\rightleftharpoons PA_n \end{aligned}\right\} \quad \cdots\cdots\cdots (3)$$

These equilibria may be represented in equations defining their equilibrium constants.

$$(PA)/(P)(A) = k_1 \quad \cdots\cdots\cdots (4)$$

$$(PA_2)/(PA)(A) = k_2 \quad \cdots\cdots\cdots (5)$$

$$(PA_i)/(PA_{i-1})(A) = k_i \quad \cdots\cdots\cdots (6)$$

$$(PA_n)/(PA_{n-1})(A) = k_n \quad \cdots\cdots\cdots (7)$$

Excellent reviews on the theory of multiple equilibria are available in literature[11]. It can be shown on purely statistical considerations that the equilibrium constants k_1 ------k_n , are not independent but bear the following relationship to each other.

$$k_1 = nk , \quad \cdots\cdots\cdots (8)$$

and

$$k_i = \frac{(n-i+1)}{i} k \quad \cdots\cdots\cdots (9)$$

k is the intrinsic association constant representing the affinity of the functional group for a metal ion. The factor $\frac{n-i+1}{i}$ is a statistical factor indicating that the probability of

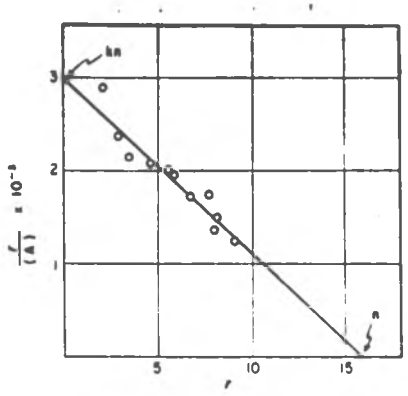


Fig. 1 : The binding of Calcium ions by casein (11)

a metal ion combining with a protein, having n identical sites, is n times larger than would be the case if the protein had a single reactive site.

A convenient measure of the extent of binding of A with a protein is the quantity

$$\frac{\text{moles bound A}}{\text{moles total protein}} = r \quad \ldots \quad (10)$$

It can be easily shown that

$$r = \frac{k_1A + 2k_1k_2A^2 + \cdots + i(k_1 \cdots k_i)A^i + \cdots + n(k_1k_2 \cdots k_n)A^n}{1 + k_1A + k_1k_2A^2 + \cdots + (k_1k_2 \cdots k_i)A^i + \cdots + (k_1k_2 \cdots k_n)A^n} \quad \ldots \quad (11)$$

which may be reduced to

$$r = \frac{nk(A)}{1+k(A)} \quad \ldots \quad (12)$$

or

$$\frac{1}{r} = \frac{1}{nk} \cdot \frac{1}{(A)} + \frac{1}{n} \quad \ldots \quad (13)$$

or

$$\frac{r}{(A)} = nk - kr \quad \ldots \quad (14)$$

Thus a plot of $\frac{1}{r}$ vs $\frac{1}{(A)}$ should give a straight line with a slope $\frac{1}{nk}$ and an intercept $\frac{1}{n}$. Similarly, a plot of $\frac{r}{(A)}$ vs r should also give a straight line with the intercepts nk and n. Such a plot is illustrated in Fig.1 for the binding of calcium ions by casein.

(III)

Electrostatic Interaction

If charged ions are bound by a protein, the first

bound ion will reduce the affinity of the protein for the second oncoming ion purely due to electrostatic repulsion. Under these conditions, equation 9 and consequently equation 13, are no longer valid. Two methods have been devised to take into account the electrostatic interaction factor in the interaction of proteins with small ions. Thus, according to Klotz and coworkers[12],

$$\Delta F^{\circ} = -RT \ln \frac{k_{i-1}}{k_i} = -RT \ln \left[\frac{n-(i-2)}{n-(i-1)} \cdot \frac{i}{i-1} \right] + \Delta F^{\circ}_{elec.} \quad \ldots \quad (15)$$

where $$\Delta F^{\circ}_{elec.} = - \frac{N(Z')^2 e^2}{D} \left[\frac{1}{b} + \frac{K}{1+Ka} \right] \quad \ldots \ldots \quad (16)$$

Z' is the charge on the metal ion, b the radius of the protein molecule, a the distance of closest approach of the metal ion to the protein, and K the familiar function of the ionic strength in the Debye-Huckel equation. ΔF^{o} elec. may be calculated readily. For the evaluation of successive association constants, one association constant must be known. The usual procedure is to calculate k_1 by an analysis of data in terms of eqn. 13 or eqn. 14 and to evaluate the successive association constants from eqn. 15. Once the successive association constants are known, values of r may be calculated from eqn. 11. Successive approximations may be employed in k_1 till a good fit with the experimental data is obtained.

The second method developed by Scatchard[13] leads to the conclusion that, for an isoionic protein with more than 4

equivalent sites,

$$\frac{r}{(A)}\, e^{2wr} = kn - kr \qquad \ldots\ldots\ldots (17)$$

where $$w = \frac{N(Z')^2e^2}{2RTD}\left[\frac{1}{b} - \frac{K}{1+Ka}\right] = \frac{\Delta F^{o}_{elec.}}{2RT} \qquad \ldots\ldots\ldots (18)$$

Thus a graph of $\frac{r}{(A)}\, e^{2wr}$ vs r should give a straight line from the intercepts of which both k and n can be evaluated. Eqn. 17 is valid only for isoionic protein but no such restriction is imposed in the evaluation by eqn. 16.

The intrinsic association constants thus obtained may be compared with the first association constant of the metal ion with the various constituent amino acid residues to reveal the nature of the functional group responsible for binding the metal ion.

(IV)

The Interaction of Serum Albumin with Metal Ions

It has been shown above how the binding data may be processed for the evaluation of intrinsic affinity constants and the number of reactive sites available on a protein molecule. We will now review the various methods used for obtaining such binding data and the results so far obtained with bovine and human serum albumins. It may be mentioned at the outset that bovine and human serum albumins are practically identical in so far as their molecular size, shape and amino acid composition are concerned[14] (See Table III).

A. Equilibrium Dialysis : The equilibrium dialysis technique has been used widely in the study of the interaction of proteins with small ions. The solutions containing a protein and a metal ion are separated by a semi-permeable membrane, such as cellophane, which is impervious to the protein but allows unhindered movement of metal ions across the membrane. If metal-protein interaction has occurred at all, the total metal concentration will, at equilibrium, be more in the protein solution than that in the protein - free solution, the difference divided by the protein concentration (Cp) being quantitatively equal to r, the number of metal ions bound per mole of the protein. The metal concentration in the protein-free solution will represent the concentration of the free metal ions, (A), in equilibrium with the metal-protein complex.

(i) Equilibrium Dialysis in buffered solutions :

Buffer solutions have been used largely for a study of the interaction of proteins with anions. A study of metal-protein interactions has, however, been limited to the work of Klotz and coworkers on the binding of cupric[15,16] and calcium ions[17] by bovine serum albumin. It was found that the binding power of bovine serum albumin for cupric ions increased with pH (acetate buffers of pH 4.0, 4.8 and 6.5 were used). The binding data could not, however, be explained in terms of the statistical factors alone. The calculated correction for electrostatic interaction ($F_{elec.}$ being 186 cals./mole at pH 4.8, ionic strength 0.06, and 125 cals./mole at pH 6.5, ionic

strength 0.20 at 0°C) likewise proved to be inadequate. It was, however, found that with an arbitrary $\Delta F_{elec.}$ value of 60 cals./mole at pH 4.8, and of ~25 cals./mole at pH 6.5 the binding data calculated from eqns 15 and 11 agreed well with the experimental. The decrease in the electrostatic interaction factor has been ascribed to the fact that the serum albumin molecule may not be spherical and that the uptake may involve the $[Cu\ Ac]^+$ ion rather than the Cu^{++} ion.

The free energy change accompanying the formation of the first complex, PCu has also been evaluated. These values together with those for the first association constant, k_1 , are given in Table IV.

Table - IV

Thermodynamics of the binding of cupric ion by bovine serum albumin (16).

pH	ΔF_1 at 0°C cal/mole	ΔF_1 at 25°C cal/mole	ΔH^0 cal/mole	ΔS^0 cal/mole/-degree	k_1 0°C	k_1 25°C
4.8	-5179	-5908	2780	29.2	1.45×10^4	2.23×10^4
6.5	-6380	-7060	1050	27.2	1.33×10^5	1.57×10^5

The enhanced ability of the albumin has been sought to be explained in terms of the increased electrostatic attraction consequent on an increase in pH. On the other hand, the

absorption spectra of copper-albumin complexes at pH values of 4.8 and 6.5 show profound differences[16] (see p. 19). These differences in absorption spectra have led to the conclusion that, at pH 4.8, the combination takes place essentially through the free carboxyl group, but that Cu...N linkage assumes an increasingly important role at pH 6.5. Esterfication of the free carboxyl groups resulted in a considerable decrease in absorption at pH 4.8 whereas, at pH 6.5, it did not. The binding of cupric ions at pH 6.5 has thus been attributed to the imidazole groups of the albumin molecule. In view of the difference in the nature of the sites responsible for binding cupric ions at pH 4.8 and 6.5, it does not seem clear how the enhanced binding at pH 6.5 can be explained solely in terms of the increased net negative charge on the protein molecule.

Fiess and Klotz[18] have studied the affinity of various proteins for the cupric ion in an acetate buffer of pH 6.5. The order of affinity, α-casein $>$ β-casein $>$ bovine serum albumin $>$ β-lactoglobulin $>$ lysozyme, could not however be related to the amino acid composition, but was found to parallel the isoelectric points of these proteins. It was thus suggested that the binding may arise out of electrostatic factors alone and that no special configurations may be required in the protein molecule in order that it bind cupric ions.

The binding of calcium ions by bovine serum albumin has been studied by Katz and Klotz[17] who ascribed it to the

free carboxyl groups of the protein molecule. The binding was found to be independent of temperature.

Fiess[19] has studied the binding of the cobaltous ions by α-casein, bovine serum albumin and pepsin at pH 6.5. He found that α-casein bound more than albumin and pepsin did not bind the cobaltous ions.

(ii) Equilibrium dialysis in unbuffered solutions : The distinguishing feature of studies in unbuffered as against those in the buffered systems, is that the data can be evaluated and interpreted in terms of a competition between a metal and the hydrogen ions for reaction with the same sites of a protein molecule. The measurement and interpretation of such competition are rendered difficult, if not impossible, in buffered solutions which are designed to yield or to absorb large amounts of hydrogen ion without indication[20]. The studies in unbuffered solutions have, so far, been limited to those of the interaction of human serum albumin with zinc[21] and lead ions[22]. The procedure consisted in dialysing an isoionic protein solution containing known amounts of dilute alkali, against the metal ion solution to which sufficient salt was added to give the desired equilibrium concentration of the medium. After equilibrium, the solutions were analysed for their metal contents, from which r and A could be evaluated. The pH of the protein solution was also measured.

Gurd and Goodman[21] have studied the binding of zinc ions by human serum albumin in 0.15M sodium nitrate at $0^{\circ}C$. They have concluded that the imidazole groups of the albumin

molecule are the primary reactive sites for binding zinc ions, and that the reaction involves a 1:1 combination between the zinc ion and the imidazole group. The intrinsic association constant ($\log k^{o} = 2.82$) was found to be in good agreement with the first association constant of the zinc ion with imidazole ($\log k_1 = 2.76$, see Table V).

Table - V (*)

First Association Constants of some cations with acetate, imidazole and ammonia

Cation	Log k_1		
	Carboxyl	Imidazole	Amino
Cu^{+2}	1.7	4.4	4.2
Zn^{+2}	1.0	2.8	2.4
Cd^{+2}	1.3	2.8	2.7
Ni^{+2}	-	3.27 (†)	2.8
Co^{+2}	-	-	2.1
Pb^{+2}	2.0	-	-
Ca^{+2}	0.5	-	-0.2

* Taken from Scatchard et al in Chemical Specificity in Biological Interactions. Acad.Press Inc. N.Y. (1954) p.215.

† Ref. (30).

The binding of lead ion (Pb^{++}) by human serum albumin has been studied by Gurd and Murray[22] in 0.15M sodium

perchlorate at 0°C. It was found that as many as 70 plumbous ions could be bound per protein molecule. The addition of zinc ions did not effect the binding of lead indicating that the imidazole groups of the protein molecule are not the reactive sites. Furthermore, human serum albumin was found to bind considerable amounts of lead ion even at low pH values. It was therefore concluded that the carboxyl groups of the protein molecule are the primary sites responsible for binding lead ions. The apparent intrinsic association constant had a value of 32-47, which is comparable with the first association constant of lead ion with the acetate ion ($\sim$100).

B. Polarography : It is well known that the limiting current due to the reduction of a metal ion at the dropping mercury electrode may be considerably reduced by the presence of proteins. This decrease has been ascribed to complex formation[23]. The diffusion current in the absence of a protein is given by

$$i_d = C A_o \quad \ldots\ldots\ldots \quad (19)$$

where i_d is the diffusion current in microamperes, A_o the total metal ion concentration and C is the diffusion current in microamperes per unit concentration of the free metal ion determined from standard solutions and corrected to the same value of $m^{2/3}t^{1/6}$. The diffusion current, $(i_d)_p$, in the presence of a protein may be expressed as the sum of two terms[24], one for the free metal (concentration A) and one for the protein-bound metal (concentration A_o-A). Thus

$$(i_d)_p = C\left[A + \propto(A_o - A)\right] \quad \cdots\cdots\cdots \quad (20)$$

$$\frac{(i_d)_p}{i_d} = \frac{A + \propto(A_o - A)}{A_o} \quad \cdots\cdots\cdots \quad (21)$$

where $\propto$ is a small fraction indicative of the characteristics of the reduction of the metal protein complex ion at the dropping mercury electrode.

Tanford[24] has examined the binding of Cu^{++}, Zn^{++}, Cd^{++} and Pb^{++} ions by bovine serum albumin in 0.15M potassium chloride. Keeping the total metal concentration fixed at 4×10^{-4}M and protein at 1.23%, the values of $(i_d)_p/i_d$ were plotted as a function of pH. A steep fall in the $\frac{(i_d)_p}{i_d}$ vs pH curves was observed in the pH range 5-6,— a range in which the albumin imidazole groups lose their protons. Assuming that the constant $\propto$ is known, it is possible to calculate the current ratio corresponding to 50% combination (eqn.21) and then to read off the corresponding pH from the experimental curve. The data may then be considered in terms of equilibria involving the imidazole groups and metal ions on the one hand, and the imidazole groups and hydrogen ions on the other. Thus

$$\frac{r}{(n_t - r - n_{HIm})A} = k^o e^{-2wZ_m Z_p} \quad \cdots\cdots\cdots \quad (22)$$

$$\frac{n_{HIm}}{(n_t - r - n_{HIm})a_H} = k^o_{HIm} e^{-2wZ_p} \quad \cdots\cdots\cdots \quad (23)$$

where n_t is the total number of imidazole sites, r the number of these sites covered by the metal ion, n_{HIm} the number which are protonated, Z_m the charge on the metal ion, Z_p the charge on the protein complex and w as defined by eq.18.

The exponential term is the usual electrostatic correction factor. The values of r and a_{H^+} corresponding to 50% combination are known. Similarly n_t and k°_{HIm} are also known. Making suitable assumptions in respect of Z_p, Z_m and w, it is possible to evaluate n_{HIm} from eqn.23 and consequently k°, the intrinsic association constant, from eqn.22.

The intrinsic association constants thus evaluated have log k° values of 3.7, 2.9, 2.8 and < 2.3 for Cu^{++}, Zn^{++}, Cd^{++} and Pb^{++} ions respectively. A comparison of these values with those for the first association constant of imidazole with the metal ions (Table V) suggests that the imidazole groups of the albumin molecules are the reactive sites.

The value of the constant, α, may be taken as the limiting value of $(i_d)_p/i_d$ when all the metal is protein-bound, i.e. when $A=A_o$, and can be obtained by an extrapolation of $\frac{(i_d)_p}{i_d}$ vs pH curves[24]. Another procedure would be to determine $(i_d)_p/i_d$ (at a fixed metal ion concentration) as a function of the protein concentration, the limiting value of $(i_d)_p/i_d$ being equal to α[25]. Knowing the values of $(i_d)_p/i_d$ and α, it is possible to calculate r, the number of metal ions bound per mole protein from equation (21). Equation (21) can be rearranged to give

$$r \doteq \frac{A_o\left\{1 - \frac{(i_d)_p}{i_d}\right\}}{c_p(1-\alpha)} \quad \cdots\cdots \quad (24)$$

and

$$A = A_o - rc_p \quad \cdots\cdots\cdots\cdots \quad (25)$$

It is thus possible to calculate the binding data from polarographic measurements.

From a polarographic study of zinc-serum albumin complexes in an acetate buffer (pH 6.05, ionic strength 0.05) Saroff and Mark[26] have concluded that the principal sites responsible for binding the metal ion are the imidazole groups. They have, however, obtained an 'n' value of 8 and a 'k' value of 1200 which differ markedly from those reported by Gurd and Goodman.

C. Optical absorption : The absorption spectra of metal-protein complexes have given important qualitative information as to the nature of the ligand groups responsible for metal binding. A comparison with the absorption spectra of metal complexes with those of simple molecules is often helpful. Thus if metal protein complex formation involves the free carboxyl groups of the protein, its absorption spectrum will be similar to that of the metal-acetate complex. Such indeed is the case, as referred to earlier, with the copper-serum albumin complex at pH 4.8[15]. At pH 6.5, however, Cu....N linkage predominates as is evidenced by a shift in the wave-length of maximum absorption towards lower values.[16] This shift corresponds to that observed in the copper-imidazole[27] and copper-ammine complexes[28]. The probability of the imidazole groups being the reactive centres is, however, based on other considerations, such as, the ability of these groups to dissociate at the pH in question.

The cupric ion may also react with the sulfhydral group of the serum-and mercapt-albumin molecule. The spectral band at 375 mμ has been shown, by a variety of experiments, to be due to the Cu....S linkage.(29)

The absorption spectra of copper-imidazole(27) and nickel-imidazole(30) complexes have also been studied. The complexes were found to be planar in case of the cupric ion and octahedral in case of the nickel ion. The magnetic susceptibility measurements on nickel-imidazole complexes revealed the formation of ionic complexes alone.(30)

D. E.M.F.Measurements : No e.m.f. measurements on metal-protein systems are reported in the literature. The method has, however, been used by Scatchard et al for a study of the binding of small anions by serum albumin(31).

E. Electrophoresis : Metal-protein interaction may be expected to reveal itself in a change in the electrophoretic mobility of the protein. Surprisingly enough, no such measurements have been reported in the literature. The method has, however, been used for study of the interaction of bovine serum albumin with anions, such as Cl^-, I^- and CNS^-, with methyl orange, detergents and lower fatty acids.(32)

F. The binding of Hg^{++} by serum albumin : The mercuric ion differs from all other metal ions in that it has a very high affinity for the imidazole and amino groups of proteins. However, its affinity for the sulfhydral group is the dominant

feature of its interaction with mercapt-albumin,[33] resulting under suitable conditions in the formation of a dimer PS-Hg-SP.

(V)

Enthalpy and Entropy of Binding

Many investigators have demonstrated that temperature changes do not greatly affect the extent of the binding of ions by serum albumin[15,16,32,34]. The quantitative investigations of Klotz and coworkers[15,16] have confirmed the general conclusions reached in earlier qualitative work. Thus, in the binding of cupric ions by bovine serum albumin, the extent of combination shows a small increase (see Table IV) rather than a decrease one might expect[15,16]. Consequently, the combination process must be accompanied by an absorption of heat, i.e. ΔH must be positive. The enthalpy of binding, ΔH_1^o, may be computed from the temperature coefficient of the first association constant, k_1, following the general thermodynamic relationship

$$\left(\frac{\partial \ln k_1}{\partial T}\right)_p = \frac{\Delta H_1^o}{RT^2} \quad \cdots\cdots\cdots \quad (26)$$

Furthermore, since

$$\Delta F = \Delta H - T\Delta S,$$

the corresponding entropy change may also be evaluated. The relevant values for the first cupric ion bound by bovine serum aloumin are also given in Table IV. It may oe noticed that complex formation is accompanied by a large positive entropy change. This increase in entropy has been ascribed to the

release of bound water from the hydrated cation or to some change in the shape of the protein[35].

(VI)

Studies with Amino acid Residues

It may be noticed from Table III that nearly all the amino and carboxyl groups (in α position to each other) of the constituent amino acids of the serum albumin molecule are used up in the formation of the peptide chain. It seems, therefore, that it is only the free carboxyl, imidazole and ϵ-amino groups of the albumin molecule which may react with metal ions at the physiological pH. The interaction of the individual amino acid residues with metal ions, therefore, assumes considerable significance. Metal-acetate[36] and metal-ammine[37] complexes have been studied in great detail over a number of years. The complexes of imidazole with Cu^{++},[27] Zn^{++},[27] Cd^{++},[38] and Ni^{++}[30] have, however, been studied relatively recently. Values of the relevant first association constants are given in Table V. It may be noticed that the complex forming ability of the imidazole and ϵ-amino groups are of the same order. At the physiological pH, however, it is only the imidazole groups which are nearly completely dissociated and hence become available for bonding with metal ions.

(VII)

The Effect of Buffer Ions

Buffer anions may compete with the functional groups

of the protein for binding a metal ion. Thus, Klotz and Fiess,[16] have shown that the binding of cupric ions by bovine serum albumin, at pH 6.5, was the greatest in a phosphate buffer, only slightly less in an acetate buffer and least in a citrate buffer. The complexing ability of these ions with the cupric ion itself is in the order, citrate > acetate > phosphate. Similarly, the addition of glycine depressed the binding of cupric ions by serum albumin.[16]

The existence of metal complexes in chloride[36,39] and acetate solutions[40] is well known. There is no reason to assume that these complex ions are not bound by the protein as such. Tanford, in his polarographic studies (see section IV B), has postulated the binding of MCl^+ ion by bovine serum albumin[24]. Fiess and Klotz[18] have made similar postulates in their study of the binding of cupric ions by bovine serum albumin in acetate buffers (see section IV A). An important feature of the binding of metal-buffer complexes is a reduction in the electrostatic interaction factor consequent on a decrease in the charge on the metal ion (see eqn.18). Thus, assuming that $CuAc^+$ ions are bound by serum albumin, the $\Delta F_{elec.}$ will be quarter of the value expected for Cu^{++} ions - an assumption which has been found useful in explaining the binding data. (see section IV A).

The effect of citrate and glycine on the binding of cupric ions by bovine serum albumin suggests that copper-citrate and copper-glycine complexes may not be bound by the protein

as effectively as the $CuAc^+$ complex.

The buffer anions may also be bound directly by the protein. The binding of anions by serum albumin has been studied exhaustively. It has been shown[41] that the anions are bound by the charged cationic centres (essentially, the charged ϵ-amino groups) of the protein. There is, however, no simple correlation between the maximum number of anions bound and the number of cationic centres. It has been postulated[42] that the cationic centres may not all be equivalent so that only a fraction may become available for binding anions. Similar considerations apply to the binding of organic anions and detergents[42] by proteins.

(VIII)

Protein Configuration

It may be concluded from the above discussion that the binding of metal ions with the functional groups (especially imidazole)of serum albumin involves a 1:1 interaction accompanied by the replacement of a water molecule coordinated to the metal ion by the functional group. It would thus appear that no special configurations are required in the protein molecule in order that it bind metal ions. Unfortunately, little is known of the disposition of the various amino acid resiques in the protein helix. There is however evidence to show that all the sites of a particular set of functional groups may not be identical in their binding ability. Thus it has been shown that the maximum number of small anions bound by a protein

may not necessarily correspond to the number of cationic centres as evaluated from its amino acid composition[12]. Furthermore, the binding sites may show selectivity for particular structures. A small but detectable discrimination is apparent, for example, from competition studies of Karush[43], who showed from variations in binding ability with concentration that one group of sites on albumin preferred dodecyl sulphate to certain azo-dye anions while a second set of sites preferred the latter ions. More direct demonstrations of such preference have been presented in recent comparisons of interactions with isomers. Distinct differences have been observed in the attraction of serum albumin for positional isomers[44]. Similarly, appreciable distinctions have been observed in the attraction of serum albumin for stereo-chemical isomers.[45] The effect of citrate and glycine on the binding of copper by serum albumin may, as mentioned earlier, be attributed to steric or other factors whereby, the uptake of copper-citrate or copper-glycine complexes by the serum albumin molecule is hindered. Such factors may not be operative in the uptake of $CuCl^+$ or $CuAc^+$ complex ions by albumin.

It is also probable that new sites may be uncovered on a protein due to reversible swelling or unfolding induced by strong internal electrostatic repulsions. The unanticipated increase in the ability of serum albumin to bind anions at alkaline pH may possibly be attributed to this effect[46]. It is also probable that anion binding may in itself lead to

structural changes in the protein whereby more reactive sites may become available.[45] Such a mechanism may account for the continued failure of all attempts to find an actual plateau in graphs of the number of ions bound against the free ion concentration.

A further factor influencing metal-protein interactions may be the extent of hydrogen bonding through the functional groups of the protein. An explanation of the special binding affinity of serum albumin may possibly be the relatively low content of hydroxy amino-acids in this protein as compared to a non-binding protein such as γ-globulin. The basic assumption in this explanation is that -OH side chains form hydrogen bonds with other side chains containing $-COO^-$ or $\geqslant NH^+$ groups. This internal bonding may reduce the number of polar side chains available for reaction with ions, the extent of the reduction being determined by the number of -OH groups as compared to that of the carboxyl and amino groups present in the protein molecule.[47]

(IX)

It may be concluded from the above discussion that the interaction of serum albumin with the cupric, zinc or cadmium ions takes place through the imidazole groups of the protein. These conclusions find further support from a study of the complexes between imidazole and these metal ions. In view of the fact that nickel[30] and cobaltous ions form strong complexes with imidazole (Co^{++} ion forms strong complexes

with histidine[48], presumably with imidazole as well), it is reasonable to expect that these ions should also react with the albumin-imidazole groups.

If copper, zinc and cadmium ions react with the albumin imidazoles alone, the electrophoretic mobilities of the metal-protein complex ions should be comparable under given conditions. It is not unreasonable to expect, however, that the carboxyl groups may compete with the imidazole groups especially as the number of protein-bound metal ions is increased.

With a view to obtain some information on the above aspects of metal-protein interactions, it was considered desirable to study the binding of copper, zinc, cadmium, cobalt and nickel ions by bovine serum albumin under identical experimental conditions. Corresponding measurements with modified serum albumins were also made to study the effect of the blocking of various functional groups on the metal-protein interaction. Equilibrium dialysis, polarographic, optical absorption and electrophoresis techniques were used for the purpose. Buffer solutions were used to maintain identical experimental conditions. It would have been difficult, for example, to make mobility measurements under comparable conditions in unbuffered solutions. It was also hoped that, with the buffered systems, it might be possible to obtain binding data covering a wide range of metal-ion concentrations. Such data might be helpful in discerning the effect, if any, of metal binding on protein configuration, or alternatively, on the availability of further functional groups.

In what follows, we have studied the interaction of copper, zinc, cadmium, cobalt and nickel ions with crystalline bovine serum albumin and its modified derivatives in an acetate buffer of pH 6.5, ionic strength 0.20.

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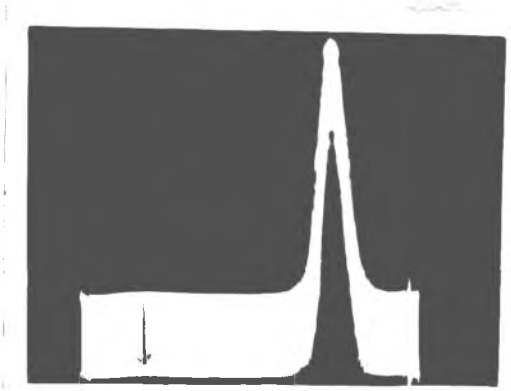

Fig. 2 : Electrophoretic Pattern of Bovine Serum Albumin (1% soln. in acetate buffer pH 6.5 & μ = 0.20. Period of electrophoresis 180 mins. field strength 2.3 volts per cm.) ↓ indicates the position of the initial boundary.

: C H A P T E R - I :

M A T E R I A L S

1. Native Bovine Serum Albumin (NBSA) :

Crystallized bovine serum albumin was an Armour product, Lot no.M 66909. A polarographic examination did not indicate any detectable amounts of metallic impurities. It was electrophoretically homogeneous in an acetate buffer of pH 6.5 and ionic strength 0.20 (Fig.2) and had a mobility of -3.12×10^{-5} cm^2/volt./sec. This value corresponds to a net charge of approximately -16 (electron units), calculated on the basis of a 0.2×10^{-5} cm^2/volt./sec. change in mobility per unit change in charge[32]. Solutions of the albumin were made by direct weighing, corrections being made for its moisture content. The moisture content was found to be 6.6%, as determined by drying to constant weight at 110°C.

2. Esterified Bovine Serum Albumin (EBSA) :

Serum albumin was esterified according to the method of Fraenkel-Conrat et al[7]. 5 g. of albumin were suspended in 500 ml of anhydrous methyl alcohol. The suspension was made 0.1N with respect to hydrochloric acid by the addition of 4.0 ml of acetyl chloride dissolved in 20 ml of methyl alcohol. The albumin dissolved readily. The solution was left at 15°C for 24 hours with occassional shaking. The precipitated ester was centrifuged at 0°C and washed thrice with ice-cold methyl alcohol. The sediment was dissolved in water, dialysed at 0°C

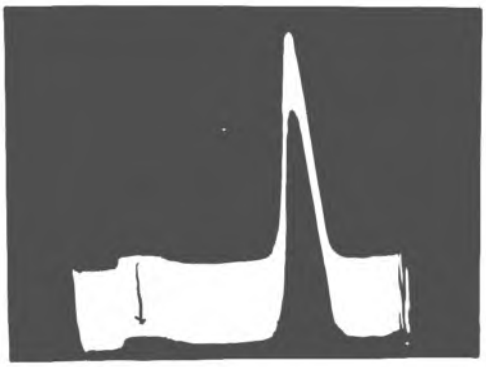

Fig. 3 : Electrophoretic Pattern of Esterified Bovine Serum Albumin (1% soln. in acetate buffer pH 6.5 & μ = 0.20. Period of electrophoresis 120 mins.; field strength 1.7 volts per cm.). ↓ indicates the position of the initial boundary.

to remove electrolytes and finally lyophilized.

The ester was analysed for its methoxy groups by the method of Niederl and Niederl[49]. The analysis showed that all the hundred and odd free carboxyl groups of the albumin molecule had been esterified. The material was electrophoretically homogeneous in the acetate buffer, pH 6.5, (Fig.3) and had a mobility of $+6.02 \times 10^{-5}$ cm^2/volt./sec, corresponding to a net charge of +30 (electron units). It would appear from the mobility of EBSA as compared to that of NBSA that only 46 carboxyl groups of the albumin molecule have been esterified as against the analytical value of about hundred and six. This discrepancy has been attributed by Saroff et al[50] to a splitting of the esterified derivative into two nearly symmetrical halves. It is our opinion, however, that the discrepancy may possibly be explained in terms of an increased binding of acetate ions by the highly positively charged esterified, derivative (see General Discussion).

The ester had a moisture content of 8.0%. It dissolved relatively slowly in the acetate buffer and rapidly in distilled water. Stock solutions were prepared in distilled water and appropriately diluted with a buffer solution of requisite concentration to give a 1% solution in the acetate buffer. (The solutions, even when kept at $0^{\circ}C$, showed a slight tendency for hydrolysis and this was considerably reduced by keeping them in a deep-freeze).

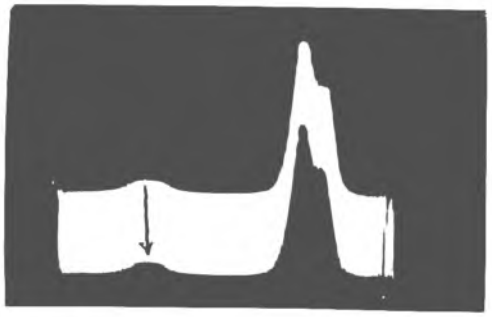

Fig. 4 : Electrophoretic Pattern of Acetylated Bovine Serum Albumin. (1% soln. in acetate buffer pH 6.5 & μ = 0.20. Period of electrophoresis 65 mins; field strength 2.0 volts per cm.). ↓ indicates the position of the initial boundary.

3. Acetylated Bovine Serum Albumin (ABSA) :

Serum albumin was acetylated by the method of Fraenkel-Conrat et al[6]. 5 g. of the albumin were dissolved in 100 ml of half saturated sodium acetate solution cooled in an ice bath. 12.5 ml of acetic anhydride were added to this solution drop by drop with constant stirring. The precipitated material was centrifuged at 0°C, suspended in distilled water and dialysed to remove dissolved electrolytes. As the electrolytes dialysed out, the product went into solution. On completion of dialysis, the solution was filtered and lyophilized.

It has been shown that acetic anhydride is specific only for the free α - & ϵ - amino groups[51]. It may, therefore, be assumed that other groups, such as the hydroxyl, the imidazolium and guanidinium are not affected. The acetylated derivative was analysed for its free amino groups by the Van Slyke nitrogen estimation as modified by Baddiley et al[52]. A value of 12 was obtained as against 68 for NBSA. The albumin had, therefore, been acetylated to the extent of 83%. The moisture content of the lyophilized product was found to be 8.2%.

The acetylated derivative was found to be electrophoretically imhomogeneous in the acetate buffer of pH 6.5 (Fig.4). Two components were observed : a slow one, constituting about 60% of the total, had a mobility of -7.60×10^{-5} cm^2/volt/sec., and a fast one, constituting the rest, had a mobility of -8.60×10^{-5} cm^2/volt/sec. The mobility computed from the

first moment of the entire gradient curve had a value of -7.87×10^{-5} cm^2/volt/sec. It has been concluded by Baddiley et al[52] from ultracentrifugal studies that the minor component has a higher molecular weight than the native albumin.

4. Buffer Solution. :

All studies, unless otherwise stated, were made in an acetate buffer solution of pH 6.5 and ionic strength 0.20. The buffer solution was prepared from Analar sodium acetate and acetic acid. This solution invariably gave a small polarographic wave at $E_{\frac{1}{2}} = -1.0$ volt, thus interfering seriously with the studies involving zinc ions ($E_{\frac{1}{2}} = -1.02$ volt.). In this and other cases, where the slight heavy metal impurity was likely to affect the results, buffer solution was prepared by adding Analar acetic acid to a solution of Merck sodium-hydroxide and adjusting the pH to 6.5. This solution did not contain any detectable metallic impurity.

5. Analar cupric chloride and cupric acetate, Merck zinc sulphate, Zinc acetate, Cadmium acetate and Cobalt chloride were used as the source of metal ions in these studies.

6. Glycine and histidine (monohydrochloride) were B.D.H. recrystallized materials used without further purification.

-o-o-o-

: C H A P T E R - II :

OPTICAL ABSORPTION & EQUILIBRIUM DIALYSIS

The binding of copper, zinc, cadmium, cobalt* and nickel ions by bovine serum albumins.
(Acetate buffer, pH 6.5, ionic strength 0.20).

-: Experimental :-

Optical Absorption : The absorption measurements were made in a 2.0% protein solution in the standard acetate buffer. The concentration of the metal ion was 0.005M for Cu^{++}, 0.05M for Co^{++}, and 0.05M for Ni^{++}. With the esterified derivative, however, a 1.0% solution was used (a 2% solution had considerable opalescence) with a corresponding reduction in the concentration of the metal ion studied. Protein solution without the metal ion were used as blanks. The measurements were made at room temperature with a Beckmann Quartz Spectrophotometer (model DU) using 1 cm. cells.

Equilibrium Dialysis : Dialysis bags prepared from Visking sausage tubing were washed with boiling distilled water. 10 ml. of 1%protein solution (in the standard buffer) were introduced into the dialysis bag. 10 ml. of the buffer solution containing

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* A note on the binding of the cobaltous ion by bovine serum albumins has been published (J.Am.Chem.Soc., 74, 4867,(1954). The bovine serum albumin used was a gift from the (late) Dr. E.J.Cohn.

various amounts of the metal ion under investigation were introduced into a test tube made conical at the bottom end. The dialysis bag, tied at the top and with a string, was introduced into the test-tube so that the level of the solutions on either side of the bag was the same. The test-tube was then stoppered with a rubber stopper and placed in position in a wooden rack fitted over a water thermostat in such a manner as to maintain the solutions at the thermostat temperature. The rack was given a to and fro movement by a suitable mechanical arrangement. A small polystyrene bead placed in the dialysis bag also assisted in stirring the protein solution. A period of 24 hours was found to be sufficient for the dialysis to proceed to equilibrium. The equilibrium solutions were then analysed for their total metal ion content, the difference in concentration divided by the protein concentration being a quantitative estimate of r, the number of metal ions bound per protein molecule. No corrections were made for the Donnan effect.

0.5% protein solutions were used for studies involving the binding of copper ions by the serum albumins. The binding of copper and cobalt ions was studied at 25 ± 0.1°C and that of zinc and cadmium ions at 30 ± 0.1°C.

Estimation of copper : The concentration of copper ions was determined by the method of Woelfel[53] using carbon disulfide and diethanolamine as the coloring agents. 4.0 g. of diethanolamine and 1.0 ml. of carbon disulfide were dissolved

separately in 200 ml. lots of dry methanol and mixed in equal proportions, just before use, to give the reagent solution. To an aliquot portion of the solution to be analysed, 1.0 ml. of the reagent solution was added and the solution made to a known volume with an acetate buffer of pH 5.0. The buffer medium of pH 5.0 was necessary for an optimum development of the color. The yellow color produced had a maximum absorption at 430 mμ . Absorption readings were taken against blanks containing the reagent and the buffer solution alone. Beer's law was found to be valid upto at least 4 ppm. The colorimetric standardization curve was obtained with known concentrations of copper ions. The presence of the protein did not interfere with the estimation.

Estimation of cobalt : Cobalt was estimated by the method of Yoe and Barton[54] using sodium β -nitroso-α-naphtholate as the coloring agent. The reagent solution was prepared by dissolving 0.1 g. of β -nitroso- α -naphthol in 20 ml. of boiling water containing 1 ml. of molar sodium hydroxide, and diluting the solution to 200 ml. after cooling. The procedure used for the development of color was as follows : To a portion of the solution to be analysed, 1.3 ml. of 3M ammonium citrate and 1.3 ml. of 2M ammonium hydroxide were added. The solution was then diluted to 20 ml. with distilled water. 1.0 ml. of the reagent solution was then added and the volume made to 25 ml. A wine red color with an absorption maximum at 550 mμ was thus developed. Absorption readings were taken against

a blank solution containing the reagent, ammonium citrate and ammonium hydroxide only. Beer's law was found to be valid upto 1.0 ppm. At larger concentrations of the metal ion, however, the cobalt-reagent complex tended to precipitate, making the absorption measurements unreliable.

It was found that the presence of the protein did not interfere with the estimation of the metal. On the other hand, it had a distinct advantage in that the protein stabilized the metal-reagent complex thus extending the range of estimation. 2.0 ml. of the reagent solution were, however, required for a complete development of the color.

The colorimetric standardization curve was drawn from standard solutions prepared by dissolving pure cobalt metal in nitric acid, evaporating the excess acid and making to known volumes with distilled water.

Estimation of zinc and cadmium : As the concentration of the metal ions in the equilibrium dialysed solutions containing the protein cannot be determined polarographically (see Chapter III), blank runs were made alongside the experimental runs. The binding data were evaluated from a polarographic analysis of the equilibrium-dialysed protein-free solution in the experimental run, and of the blank solution.

Zinc and cadmium were estimated polarographically using a Tinsley Polarograph (Type 12) with a conventional H-type cell. One limb of the cell formed the saturated calomel half-cell, and was connected to the other limb containing the

experimental solution (about 7 ml.), through a sintered glass disc and an agar bridge. The limbs were joined through a GG joint. The experimental limb could thus be detached and cleaned satisfactorily. The cell was maintained at 30 ± 0.1°C.

0.01% gelatin was used as a maximum suppressor wherever necessary. The buffer itself acted as the supporting electrolyte. Oxygen-free nitrogen was bubbled through the solutions to remove dissolved oxygen. The polarograms were then recorded. The metal ion concentration could thus be evaluated from the measured diffusion current with a knowledge of the constant C (see eqn.19 p. 16).

The constant C was evaluated from a polarographic analysis of standard solutions of zinc sulfate and cadmium acetate in the standard buffer. The standard solutions were prepared by suitably diluting a stock solution the metal ion concentration of which was estimated gravimetrically (zinc as zinc ammonium phosphate and cadmium as cadmium sulfate). The diffusion current was found to be proportional to the concentration of the metal ion. The constant C had a value of 8.99 μA per millimole of zinc ion per litre (flow rate of mercury, m=3.26 mg. per second, drop time, t=2.3 sec per drop at $E^{\frac{1}{2}}$= -1.02 volt), and of 7.99 μA per millimole of cadmium ion per litre (m=2.90 mg. per second, t=2.67 seconds per drop at $E^{\frac{1}{2}}$= -0.60 volt) at 30°C. The corresponding values calculated from the Ilkovic equation were 8.59 and 8.11 for the zinc and the cadmium ion respectively.

Results and Discussion

Absorption Spectra : The absorption spectra of copper, cobalt

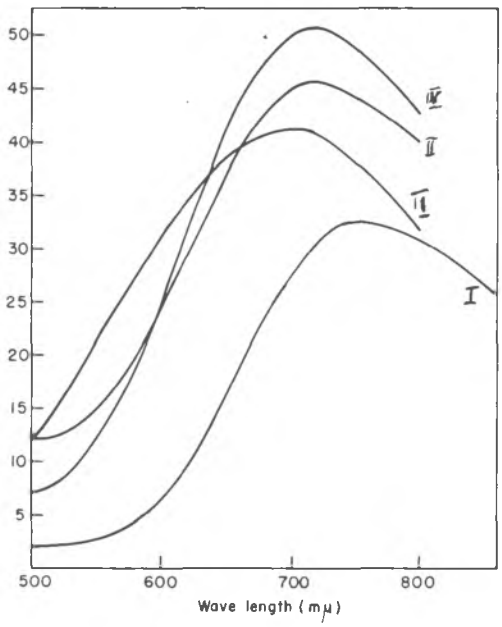


Fig. 5 : Absorption spectra of Copper-Albumin complexes in acetate buffer pH 6.5 & μ = 0.20);

I 0.005 M $CuCl_2$;

II 0.0025 M $CuCl_2$ + 1% EBSA;

III 0.005 M $CuCl_2$ + 2% NBSA;

IV 0.005 M $CuCl_2$ + 2% ABSA.

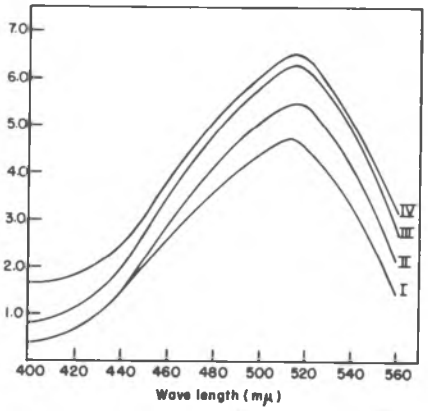


Fig. 6 : Absorption spectra of Cobalt-Albumin complexes in acetate buffer (pH 6.5 & μ = 0.20).

I 0.05 M $CoCl_2$ in distilled water;

II 0.05 M $CoCl_2$ in the buffer;

III 0.05 M $CoCl_2$ in the buffer + 2% NBSA;

IV 0.05 M $CoCl_2$ in the buffer + 2% NBSA.

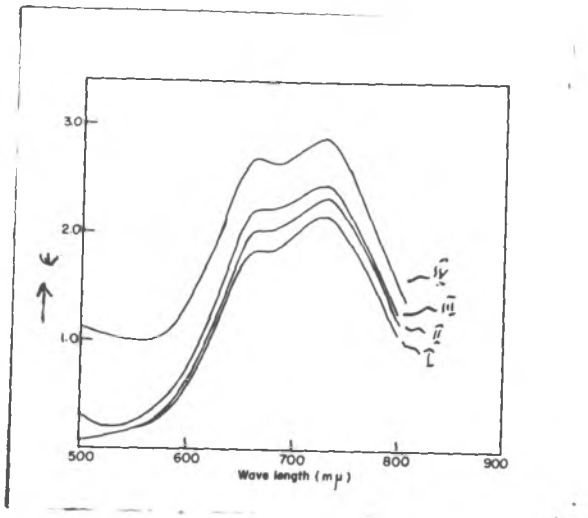


Fig. 7 : Absorption spectra of Nickel-Albumin complexes in acetate buffer (pH 6.5 & μ = 0.20).

I 0.05 M $NiCl_2$ in distilled water;
II 0.05 M $NiCl_2$ in acetate buffer;
III 0.05 M $NiCl_2$ in buffer + 2% NBSA;
IV 0.05 M $NiCl_2$ in buffer + 2% ABSA.

and nickel ions alone, and in the presence of the serum albumins are given in Figs. 5,6 and 7 respectively. The spectra of Fig.5 are similar to those reported by Klotz and Riess[16]. It is evident from Fig.5 that the absorption increases in the order ABSA > NBSA > EBSA, and corresponds to the binding abilities of these proteins for the cupric ion. The wavelength of maximum absorption is also shifted towards lower wavelengths, the shift being of the same order for the three proteins (Fig.5). This behaviour is in contrast to the observations of Klotz and Riess who reported that, whereas a 66% esterified serum albumin led to an increase in absorption of the same order as for the native albumin, the absorption peak was shifted to even shorter wavelengths.

The absorption spectra of copper - albumin systems suggest, therefore, that bonding occurs with the nitrogen-containing groups, presumably the imidazole groups, of the serum albumin molecule. The absorption spectra of the cobalt-albumin systems (Fig.6) are, however, markedly different in that they exhibit only an increased absorption without any noticeable shift in the wavelength of maximum absorption. Furthermore, the addition of the esterified derivative had no effect on the absorption spectrum of the cobaltous ion. It would thus appear that the free carboxyl groups of the protein molecule are responsible for binding the cobaltous ion and that Co....N linkage is not favoured.

The absorption spectra of nickel-albumin systems (Fig.7) are similar in every respect to those of the corresponding

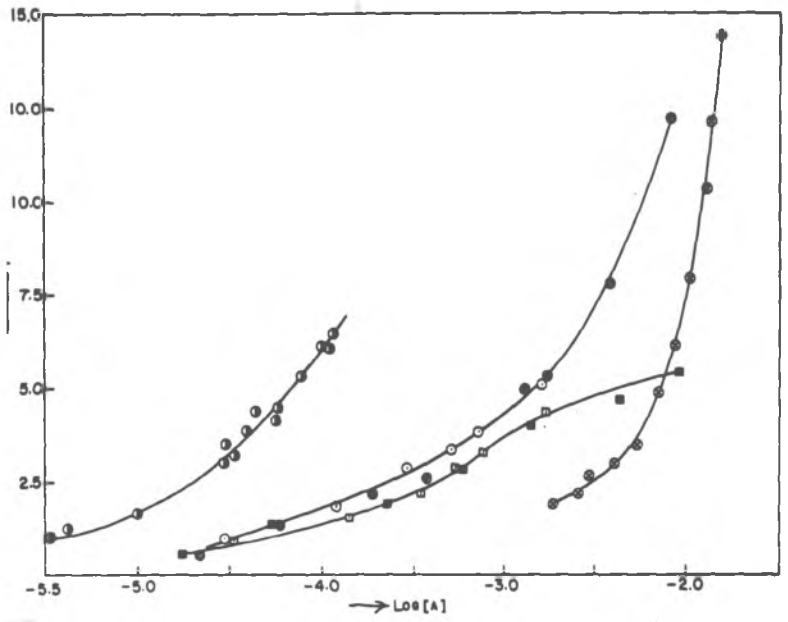


Fig. 8 : The Binding of metal ions by Bovine Serum Albumin in acetate buffer (pH 6.5, μ =0.20).

- Cu^{++} ions (Equilibrium Dialysis 25°C).
- Zn^{++} ions (Equilibrium Dialysis 30°C).
- Zn^{++} ions (Polarographic 30°C).
- Cd^{++} ions (Equilibrium Dialysis 30°C).
- Cd^{++} ions (Polarographic 30°C).
- Co^{++} ions (Equilibrium Dialysis 25°C).

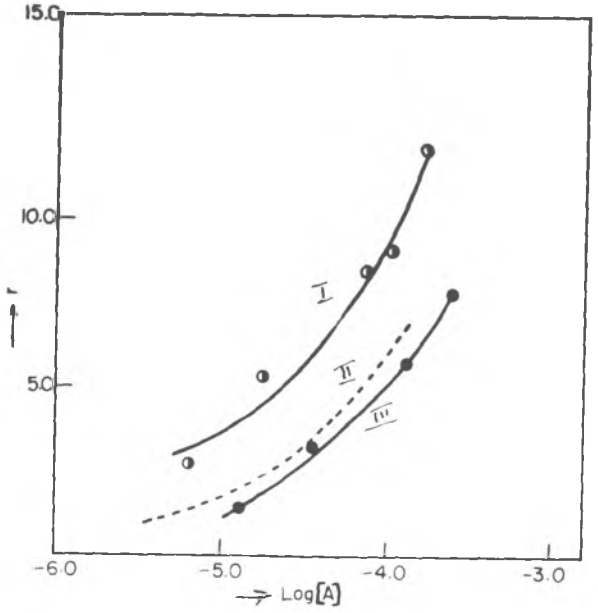


Fig. 9 : The Binding of Cu^{++} ions by the Bovine Serum Albumins ($25^{\circ}C$). (Acetate Buffer, pH 6.5, μ = 0.20).

—●— EBSA

– – – NBSA

—◑— ABSA

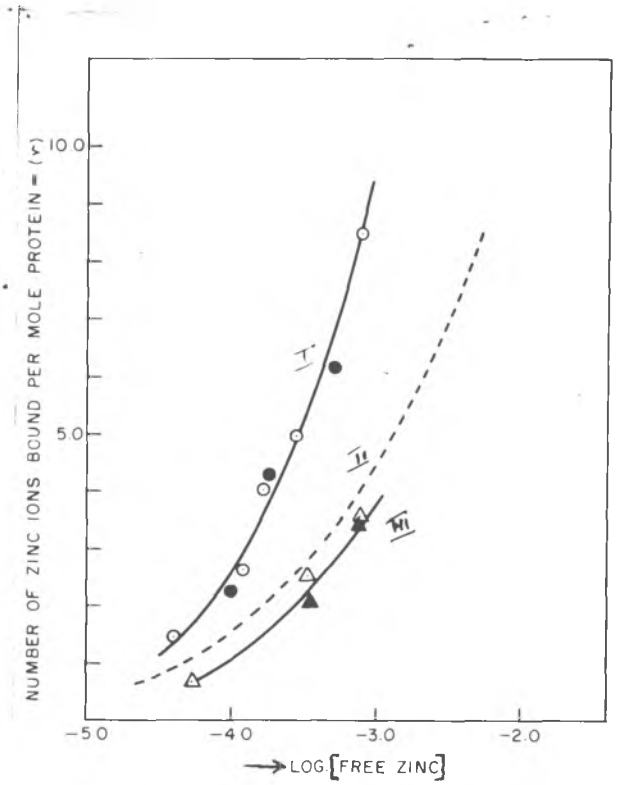


Fig.10 : The Binding of Zn^{++} ions by the Bovine Serum Albumins (30°C). (Acetate Buffer, pH 6.5, μ = 0.20).

-△- EBSA - ($ZnClO_4$)

-▲- EBSA - (Zn acetate);

--- NBSA

-●- ABSA (Equilibrium Dialysis)

-⊙- ABSA (Polarographic).

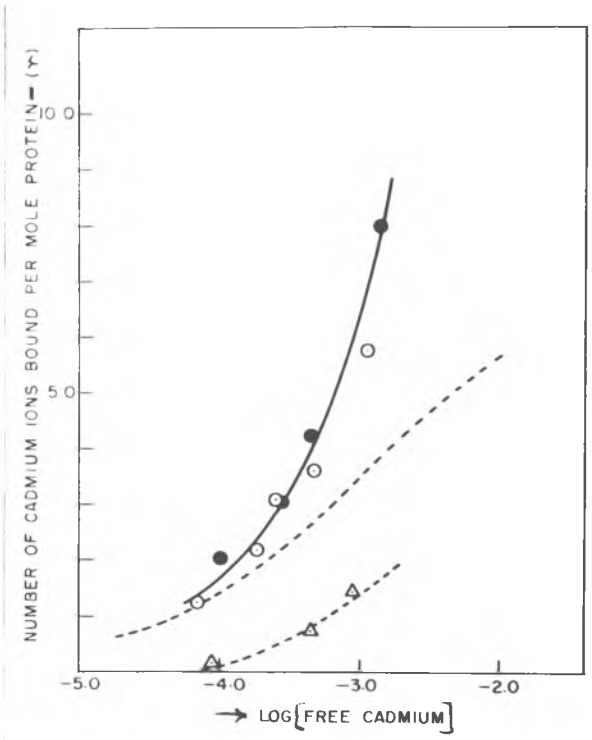


Fig.11 : The Binding of Cd^{++} ions by the Bovine Serum Albumins (30°C). (Acetate Buffer, pH 6.5, μ = 0.20).

- -Δ- BBSA
- --- NBSA
- -⊙- ABSA (Equilibrium dialysis),
- -⊙- ABSA (Polarographic).

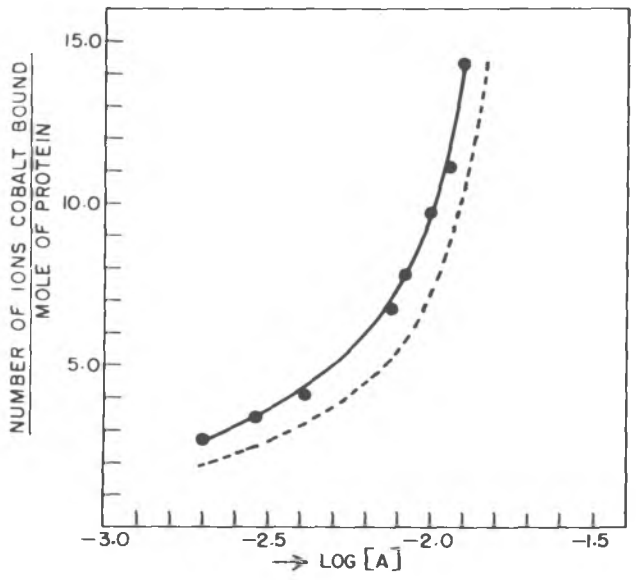


Fig.12 : The Binding of Co^{++} ions by the Bovine Serum Albumins (25°C). (Acetate Buffer, pH 6.5, μ = 0.20).

--- NBSA

—●— ABSA

cobalt-albumin systems. A comparison of Figs 7 and 6 reveals, furthermore, that the effect of the proteins on the absorption was somewhat less in the former than the latter. It seems reasonable to conclude, therefore that the ability of serum albumin to bind the nickel ion is somewhat less than that for the cobaltous ion and that the binding involves the free carboxyl groups of the albumin molecule.

Binding Data : The equilibrium dialysis data for the binding of copper, zinc, cadmium and cobalt ions by bovine serum albumin are represented in Fig.8 as plots of r vs log A. Similar plots for the acetylated and esterified derivatives are shown in Fig.9 (copper), Fig.10 (zinc), Fig.11 (cadmium) and Fig.12 (cobalt). The esterified derivative did not bind the cobaltous ion at all, emphasizing once again the role of the free carboxyl groups of the protein in binding this ion. Esterification of the serum albumin also led to a considerable decrease in the binding of the cadmium ion. The binding of the zinc and the copper ion was, however, only slightly affected by esterification, and may possibly be attributed to an increase in the net positive charge on the protein consequent on esterification. The increased binding ability of the acetylated derivative may likewise be attributed to an increased net negative charge on the protein consequent on acetylation.

An examination of the shapes of the r vs log A curves reveals some interesting features of the interaction process. Thus, for example, the curves represented in Fig.9

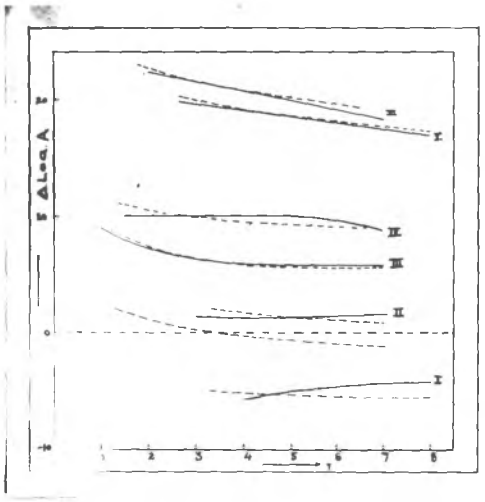


Fig.13A : Plots of Δ log A vs r (Cu-ABSA system being the reference systems).

I Cu-ABSA;

II Cu-SBSA;

III Zn- ABSA;

IV Cd-ABSA;

V Co-ABSA;

VI Co-NBSA.

Dashed curves refer to the corresponding calculated binding curves.

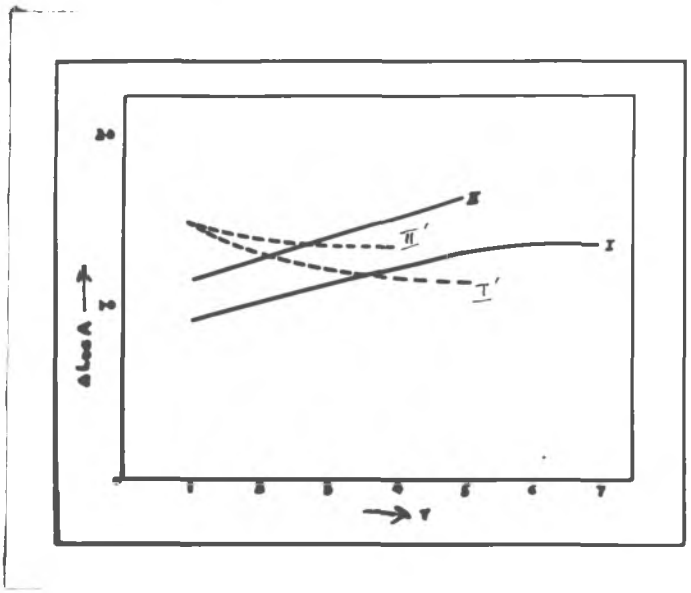


Fig.13P : Plots of Δ log A vs r (Cu-NBSA system being the reference system).

I Zn-NBSA;

II Cd-NBSA.

Dashed curves refer to the corresponding calculated binding curves.

are nearly parallel to each other; curve II may be obtained by shifting curve I along the X - axis by 0.48 (log A) units and curve III by shifting curve II in a similar manner by 0.16 (log A) units. Similarly, the curves given in Fig.12 for the cobalt-albumin systems may be regarded as nearly-parallel to each other. This state of affairs does not seem to obtain in the systems involving zinc and cadmium ions. Take, for example, Fig.10 wherein curves I and II diverge from each other as more and more metal ions are bound. An over-all picture of the relationship between the various metal-albumin systems, in so far as their r *vs* log A curves are concerned, is well represented in Fig.13. For this purpose, the system : copper - NBSA (curve II, Fig.9) was taken as a reference against which all other systems were compared. The difference in log A between any experimental curve and the reference curve at given values of r were plotted as Δ log A *vs* r (Figs. 13A & B). It may be noticed that the Δ log A *vs* r curves for the systems : Cu-ABSA, Cu-EBSA, Zn-ABSA and Cd-ABSA are nearly parallel to the reference curve except possibly in the low binding region. The corresponding curves for the systems, Zn-NBSA and Cd-NBSA, have a positive slope which is less in the former than the latter (Fig.13B). The Δ log A *vs* r curves for the systems Co-ABSA and Co-NBSA, though parallel to each other, have a negative slope. Ignoring for the present the cobalt-albumin systems wherein the free carboxyl groups of the protein molecule appear to be the reactive sites, the systems involving the binding of copper, zinc and cadmium ions do not seem to be

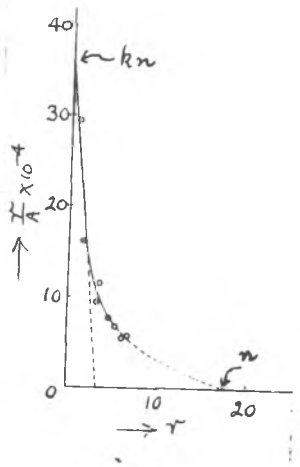


Fig.14 A : Plot of $\frac{\Gamma}{A}$ vs Γ for Cu-M33A system.

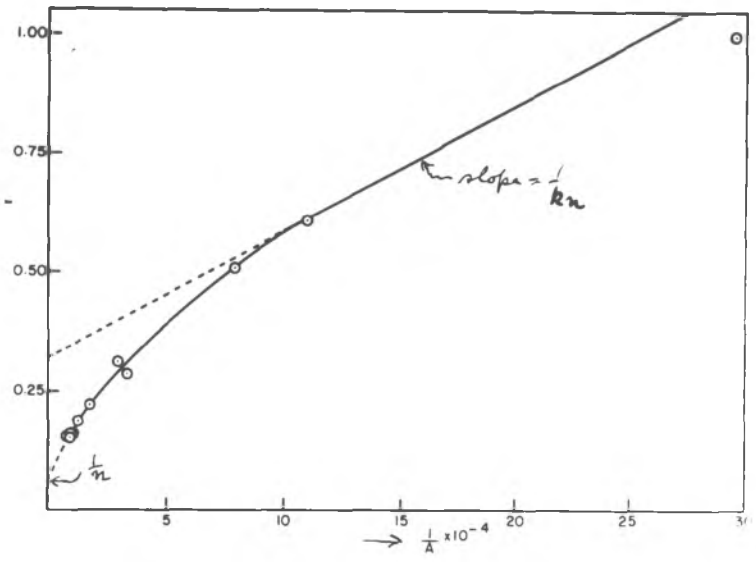


Fig.14 B : Plot of $\frac{1}{r}$ vs $\frac{1}{A}$ for Cu-NBSA system.

equivalent. Such an equivalence would be expected if only the imidazole groups of the protein were the reactive sites.

Statistical considerations : An analysis of the binding data in terms of equations 13 and 14 (p. 8) reveals that the data cannot be explained on statistical considerations alone. Representative $\frac{r}{A}$ vs r and $\frac{1}{r}$ vs $\frac{1}{A}$ plots are given in Fig.14A & B. These plots have a common feature in that they are linear for the first few metal ions bound and then deviate from linearity. The linear portion may, however, be usefully employed for an evaluation of the intercept nk (= k_1) on the Y-axis (Fig 14A). An evaluation of n (intercept on the X-axis) is rendered difficult in view of the shape of $\frac{r}{A}$ vs r curves. An extrapolation of the experimental curves (Fig.14) was, however, made for a rough evaluation of n. The values of n thus obtained may be regarded as a lower rather than an upper limit. The values of the two intercepts are given in Table VI.

Table - VI

$P + M \rightleftharpoons PM_1$

	Copper		Zinc		Cadmium		Cobalt	
	log k_1	n	log k_1	n	log k_1	n	log k_1	n
EBSA	5.17	18	4.21	18	-	-	-	-
NBSA	5.57	18	4.64	18	4.63	(7)	3.00	>50
ABSA	6.08	18	4.62	18	4.58	18	3.13	>50

It is evident from Table VI that the ability of the protein to bind metal ions, as evidenced by the values of the first association constant, changes in the order ABSA > NBSA > EBSA for a given metal ion, and copper > zinc > cadmium > cobalt (> nickel) for a given protein. In the interaction involving the zinc and the cadmium ion, however, the values of the first association constant, k_1 , are nearly the same for the native protein and its acetylated derivative. Such a result is, of course, to be expected from the differences in the shapes of the respective r vs log A curves. It seems, therefore, that the number of reactive sites or, alternatively, the ability of the sites to react with the zinc and cadmium ions may not be the same for the native albumin as for its acetylated derivative. The relatively negligible ability of the imidazole groups of the esterified derivative to react with the cadmium ion and their complete lack of ability to bind the cobaltous ion give pointed support to the above conclusions.

We do not intend, at the present stage, to discuss the significance of the number of the reactive sites, n, extrapolated from the $\frac{r}{A}$ vs r curves. An n value of 18 does, however, suggest that the 18 imidazole groups of the albumin molecules may be responsible for binding copper, zinc, and cadmium ions. Similarly, an n value of 50 or more for the cobalt-albumin systems suggests the free carboxyl groups of the protein as being essentially responsible for binding the cobaltous ion.

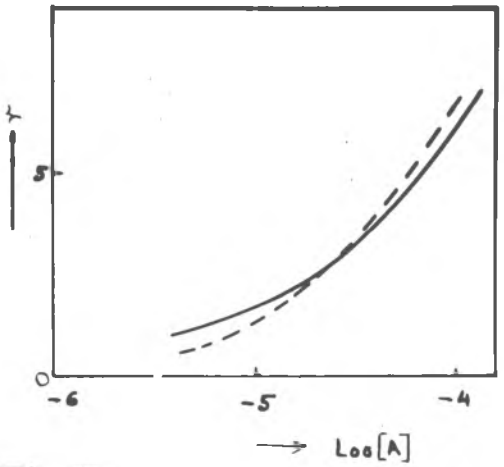


Fig.15 : Calculated and experimental binding curves for the system Cu-HSA

——— experimental;

---- Calculated.

Electrostatic Interaction : As the binding data do not fit the statistical equation, the electrostatic interaction term was also taken into account. We have followed the method developed by Klotz[12] as it involves no assumptions as to the charge on the protein molecule. With the values of k_1 and n given in Table VI and with suitable values of $\Delta F_{elec.}$ the successive association constants were calculated from eqn.15 (p 9) and the binding data from eqn.11 (p. 8). With successive approximations the value of k_1 was arbitrarily reduced to give the best possible fit between the calculated and the experimental binding data. Fiess and Klotz[18] have reported that with n = 16, $k_1 = 1.57 \times 10^5$, and a $\Delta F_{elec.}$ of about 25 cal./mole, the calculated data fitted well with the experimental data for the binding of the copper ion by serum albumin in an acetate buffer of pH 6.5. The data calculated on the above basis are plotted in Fig.15 together with the experimental data. The fit does not appear to be very good especially in the low binding region. If, however, we assume that the binding data in this region is subject to large errors, the fit may be regarded as fairly good.

We have made calculations of the type illustrated above on the following basis. An n value of 50 was assumed for the cobalt-albumin systems, and of 18 for others. The $\Delta F_{elec.}$ was calculated on the assumption that the metal uptake involved the singly charged MAc^+ ion which may be largly present in 0.2M acetate solution. Earlier workers have also made such assumptions[18,24]. We have, however, used a $\Delta F_{elec.}$ value of

40 cal. per mole (calculated value 35 cal./mole) in view of the possibility that a small fraction of the uptake may involve the M^{++} ion. The assumed values of k_1 are given in Table VII.

Table - VII

$P + M \rightleftharpoons PM_1$

Values of Log k_1

	Copper	Zinc	Cadmium	Cobalt
EBSA	5.00	3.93	-	-
NBSA	5.18	4.04	4.00	3.04
ABSA	5.69	4.54	4.20	3.20

The extent of the fit between the calculated and the experimental binding data is indicated in Fig.13A & B. The calculated curves in Fig. 13A & B are all nearly parallel to each other and slope downwards. Disregarding the low binding region, the fit may be regarded as fairly good for the systems : Cobalt - ABSA, Cobalt - NBSA, Cadmium - ABSA, Zinc - ABSA, Copper - ABSA, Copper - NBSA, Copper - EBSA (Fig.13A), and particularly bad for the systems : Zinc - NBSA, Cadmium - NBSA (and zinc - EBSA. Not shown in Fig.13B). In the latter systems, no single set of values for n, k_1 and $\Delta F_{elec.}$, howsoever varied, seemed to fit the data.

We have assumed in the above discussion that the charge on the protein molecule increases uniformly with the

uptake of metal ions. It will be shown later that, with the possible exception of the cobalt-albumin systems, such a situation does not, in fact, obtain (see Chapter IV). It will also be shown that the value of n assumed for the interaction of bovine serum albumin with copper, zinc and cadmium ions bears no relationship to reality (see p.74). The above calculations, however, serve a useful purpose in that they point out clearly (i) that the cobalt-albumin systems may be explained adequately in terms of an interaction with the free carboxyl groups of the protein and (ii) that the binding of zinc and cadmium ions by bovine serum albumin cannot be explained on the basis of a reaction with imidazole groups alone. It will be shown later that the other systems investigated by us fall into the later category (see General Discussion).

A tacit assumption has been made in the discussion outlined above that the interaction with a metal ion occurs at a single set of equivalent sites. In view of the experimental data, it seems to us that this may not be the case particularly in the interactions involving the imidazole groups of the serum albumins.

Intrinsic Association Constants : An evaluation of the apparent (uncorrected for the charge on the protein molecule) intrinsic association constant from the first association constant, k_1, (Table VI) involves a knowledge of n, the number of sites on the protein molecule available for reaction with the first metal ion. It is not unreasonable to expect, however, that

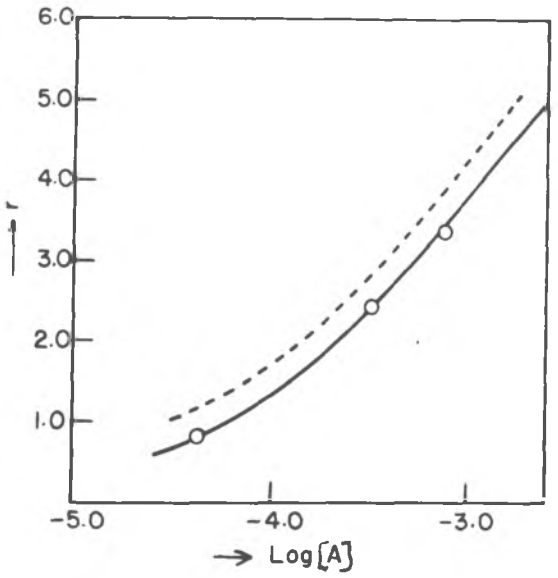


Fig.15 A : Effect of Cd^{++} ions on the binding of Zn^{++} ions by NBBA (Acetate Buffer pH 6.5 & μ = 0.20 ; Cd^{++}/Zn^{++} = 1.0).

Dashed curve refers to Zn^{++} binding in the absence of Cd^{++}

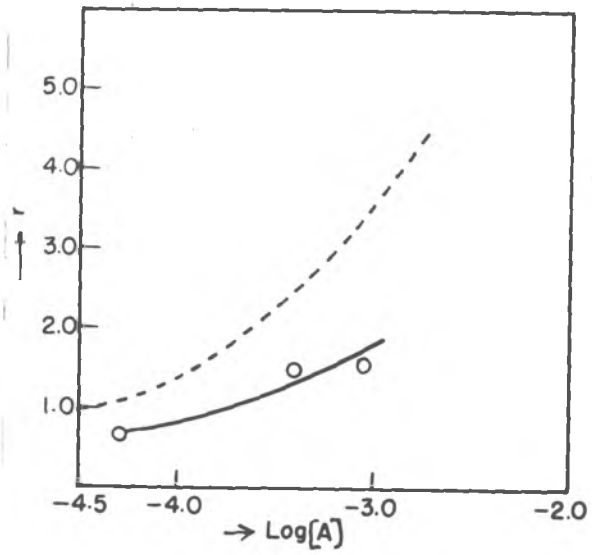


Fig.16 B : Effect of Zn^{++} ions on the binding of Cd^{++} ions by NBSA (Acetate buffer pH 6.5 & μ = 0.20 ; Zn^{++}/Cd^{++} = 1.0).

Dashed curve refers to Cd^{++} binding in the absence of Zn^{++}.

(See Text)

the number of dissociated functional groups, especially the imidazole groups, will not be the same in the serum albumin as in its derivatives even though the total number of imidazole groups (dissociated and undissociated) be the same. We shall therefore postpone an evaluation of the intrinsic association constants until after the nature of the metal-protein interactions becomes somewhat clearer than it is at present.

Competition Studies : In the competition experiments to be described below, it may perhaps be useful to keep in mind the results obtained by earlier workers to the effect that the imidazole groups of the serum albumin molecule are essentially responsible for binding copper, zinc and cadmium ions at the physiological pH.

(i) Competition between zinc and cobalt. The binding of the cobaltous ion by the bovine serum albumin was found to remain unaffected in the presence of the zinc ion. It is suggested, therefore, that the binding of cobalt and zinc ions involves different sets of sites. Such a result is, of course, to be expected in view of the experimental evidence as to the interaction of the cobaltous ion with the free carboxyl groups of the protein.

(ii) Competition between zinc and cadmium ions. Two sets of experiments were conducted. In the first set, the ratio of the total cadmium to total zinc was kept constant (at unity). The relevant r vs log A plots are shown in Fig.16A & B. It may be noticed that whereas the binding of the zinc ion is only slightly affected by the presence of the cadmium

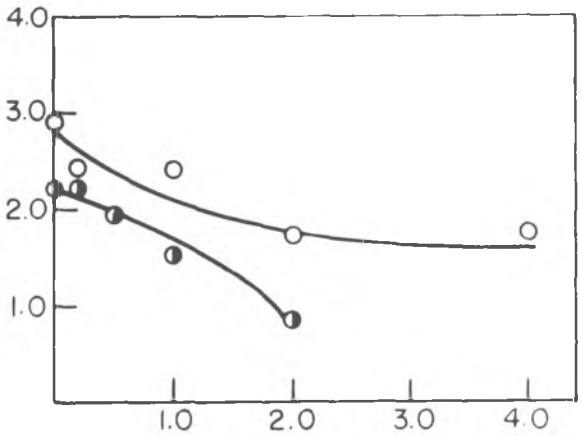


Fig.17 : Competition between Zn^{++} & Cd^{++} ions for combination with NBSA. (acetate buffer pH 6.5 & μ = 0.20).

—⊙— Zn^{++} ion concn = 5.0×10^{-4}M and Cd^{++} ion concn varied from 1.0×10^{-4}M to 5.0×10^{-3}M

—◑— Cd^{++} ion concn = 5.0×10^{-4}M and Zn^{++} ion concn. varied from 1.0×10^{-4}M to 1.0×10^{-3}M.

(See Text)

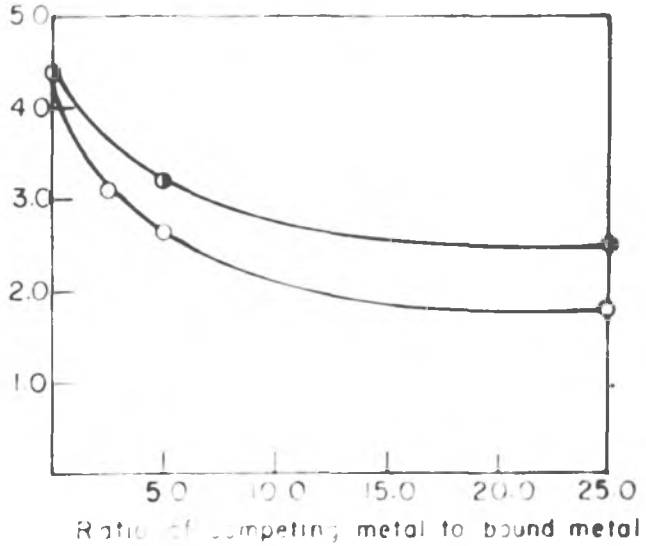


Fig.18 : Effect of Zn^{++} & Cd^{++} ions on the binding of Cu^{++} ions by NBGA (acetate buffer pH 6.5 & μ = 0.20).
(Cu^{++} ion conen = 2.0 x 10^{-4}; Zn^{++} ion conen. varied from zero to 5.0 x 10^{-3}M; Cd^{++} ion concn varied from zero to 5.0 x 10^{-3}M).

-⊙- Effect of Zn^{++} ions

-●- Effect of Cd^{++} ions

ion, that of the cadmium ion is considerably reduced especially at large metal ion concentrations. It is reasonable to expect therefore that, in a competition between zinc and cadmium ions for reaction with the functional groups of serum albumin, the successive association constants for the binding of cadmium ions are reduced much more rapidly than for the zinc ion.

In the second set of experiments, the concentration of one metal was kept constant and its binding by the serum albumin measured with varying concentrations of the other metal ion (Fig.17). It may be noticed from Fig.17 that the ability of the cadmium ion to combine with the functional groups is reduced considerably in the presence of a relatively large concentration of the zinc ion. It is therefore reasonable to conclude, in conformity with the other data presented above, that the factors governing the binding of zinc and cadmium ions by serum albumin may be somewhat different for the two systems.

(iii) Competition between copper and zinc (and cadmium) ions. The total copper ion concentration was kept constant at 2.0×10^{-4}M and that of the competing metal ion varied over the range $0 - 5.0 \times 10^{-3}$M. The results are shown in Fig.18. It may be noticed that the number of the bound copper ions is reduced appreciably in the presence of the competing metal ion. However, the effect becomes less pronounced as the concentration of the competing metal is increased.

It has already been pointed out by us (p.) that the binding data cannot be explained in terms of an interaction with the imidazole groups of the protein alone. The results

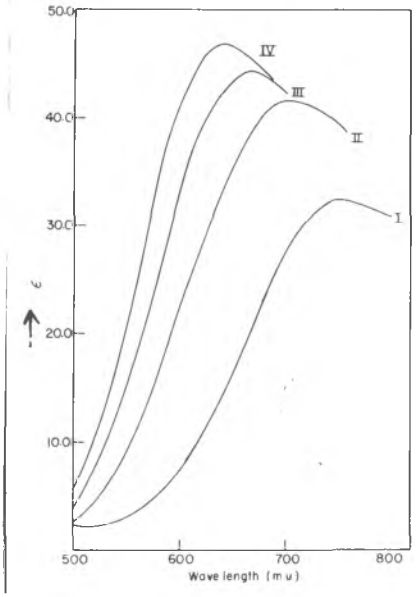


Fig.19 : Absorption spectra of copper-glycine complexes (acetate buffer pH 6.5 & μ = 0.20).

I 0.005 M $CuCl_2$;

II 0.005 M $CuCl_2$ + 0.005 M glycine;

III 0.005 M $CuCl_2$ + 0.010 M glycine;

IV 0.005 M $CuCl_2$ + 0.020 M glycine.

described in (ii) and (iii) above, fall into the same category. As a first step, we may consider the ability of the carboxyl groups of the protein to compete with the imidazole groups for binding metal ions. In view of the relatively high affinity of imidazole for copper, zinc and cadmium ions (see Table V), the uptake of the first metal ion may be expected to occur at an imidazole group of the serum albumin molecule. As more metal ions are bound, however, the participation of the free carboxyl groups of the protein in the interaction process will be expected to become increasingly pronounced. Furthermore, this participation will be expected to be in the order, Cd > Zn > Cu, opposite to that of the imidazole group. We are thus in a position, on the above basis, to explain, at least qualitatively, why the affect of zinc (and cadmium) on the binding of copper becomes less and less pronounced as the concentration of the former is increased (Fig.18). The competition between cadmium and zinc ions for interaction with bovine serum albumin (Figs.16 & 17) may be explained in a similar manner.

(iv) Competition between glycine and serum albumin.

The binding of the zinc ion by bovine serum albumin was affected but little in the presence of glycine. Glycine has, however, been reported to compete successfully with serum albumin for binding the copper ion[16]. This difference in behaviour may possibly be attributed to the nature of copper-glycine and zinc-glycine complexes under the experimental conditions. Glycine forms strong complexes with copper (Fig.19), and, as such, would be expected to compete successfully with the albumin-

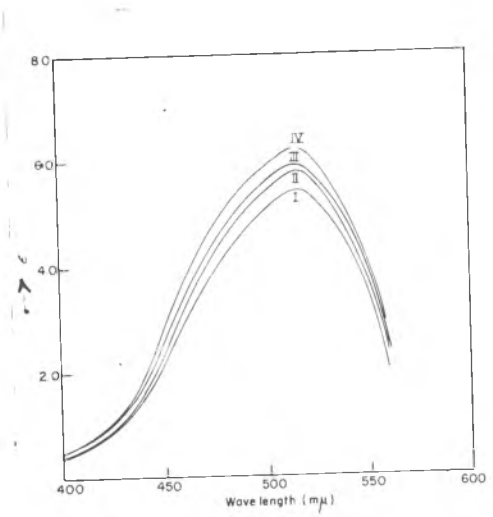


Fig.20 : Absorption spectra of Cobalt-Glycine complexes (acetate buffer, pH 6.5 & μ = 0.20).

I 0.05 M $CoCl_2$;

II 0.05 M $CoCl_2$ + 0.05 M glycine;

III 0.05 M $CoCl_2$ + 0.10 M glycine;

IV 0.05 M $CoCl_2$ + 0.20 M glycine.

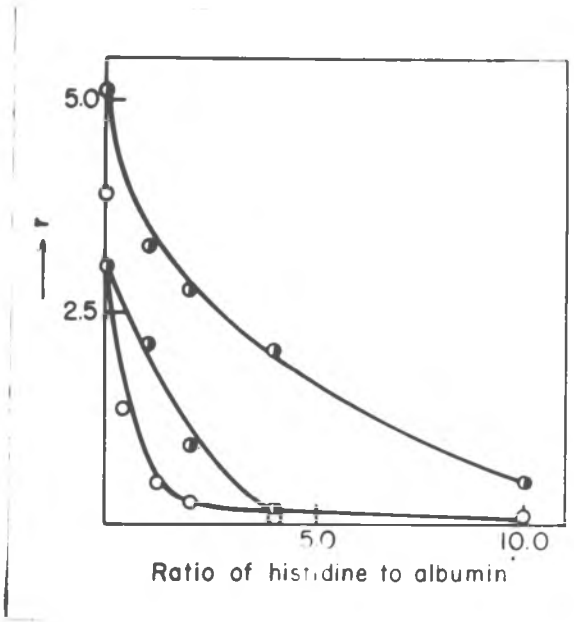


Fig.21 : Effect of Histidine on the binding of metal ions by NBSA.

-O- Cu^{++} ions; (Total concn 1.8×10^{-4} M)

-O- Zn^{++} ions. [Total concn 2.0×10^{-3} M (upper curve), 1.0×10^{-3} M (Lower curve)]

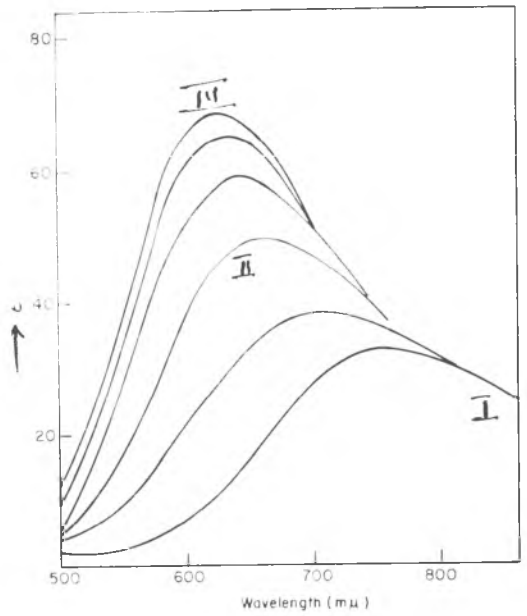


Fig.22 : Absorption spectra of Copper-Histidine complexes (acetate buffer pH 6.5 & μ = 0.20).

I 0.005 M $CuCl_2$;

II 0.005 M $CuCl_2$ + 0.005 M histidine;

III 0.005 M $CuCl_2$ + 0.020 M histidine.

imidazole. The relatively negligible effect of glycine on the ability of serum albumin to bind the zinc ion may possibly be attributed to a low affinity of glycine for the zinc ion. Whereas we have no direct evidence to support such an assumption, the absorption spectra of cobalt-glycine complexes may be of particular interest (Fig.20). A comparison of Fig.20 with Fig.19 points to a strong possibility that the cobalt-glycine complexes are of the metal-acetate type and that the α-amino group does not participitate in complex formation. It is not unlikely that zinc may also form weak complexes of the acetate type with glycine.

(v) Competition between histidine and serum albumin. Histidine was found to compete successfully with the functional groups of serum albumin for binding metal ions. The effect was more pronounced on the binding of the cupric than of the zinc ion (Fig.21). The copper-histidine complexes are exceptionally strong (Fig.22), and involve chelation through the imidazole and the α-amino group of the amino-acid[27]. On analogy with the zinc-glycine complexes (see (iv) above), we may assume that the α-amino group of histidine does not react with the zinc ion at pH 6.5. The stability of zinc-histidine complexes will, therefore, be expected to be of the same order as that of the zinc-imidazole complexes. The relative ease with which histidine can remove the copper as compared to the zinc ion from the respective metal-serum albumin complexes may possibly be explained on the above basis.

A few broad conclusions may be derived from the results reported in this chapter. They are :-

(i) that the interaction of bovine serum albumin with the metal ions investigated follows the order, Copper > Zinc > Cadmium > Cobalt (> nickel);

(ii) that the binding of copper, zinc and cadmium ions involves, in the initial stages of the interaction at least, the imidazole groups of the serum albumin molecule;

(iii) that the binding data cannot be explained in terms of an interaction with the imidazole groups alone;

(iv) that the participation of the carboxyl groups in the interaction process may be pronounced;

(v) that this participation follows the order Cd > Zn > Cu;

(vi) that the uptake of the cadmium ion becomes negligibly small if the free carboxyl groups of serum albumin were blocked;

and (vii) that the cobaltous ion is bound essentially at the free carboxyl groups of the serum albumin molecule.

-o-o-o-

-: C H A P T E R : III :-

POLAROGRAPHIC STUDIES

The Interaction of zinc and cadmium ions with bovine serum albumins.

(acetate buffer, pH 6.5, ionic strength 0.20)

It has been indicated earlier (see Introduction p.16) that the binding data can be evaluated with a knowledge of constant α and of the ratio $\frac{(i_d)_p}{i_d}$ of the diffusion currents of the metal ion with and without the presence of the protein (equations 24 and 25, p.18). Saroff and Mark[26] have shown that for the system zinc-NBSA, at pH 6.1, the binding data thus evaluated, using an α value of 0.30 agreed fairly well with that obtained from equilibrium dialysis in the low binding region only. It is our object in this chapter to find out whether or not the polarographic measurements in buffered solutions yield binding data comparable to those obtained from equilibrium dialysis and whether such measurements give any additional information as to the nature of metal-protein interactions. A knowledge of the constant α is necessary for this purpose. The only reliable determinations of this constant are those of Tanford for the systems, Cu-NBSA ($\alpha = 0$), Zn-NBSA ($\alpha = 0.10$), Cd-NBSA ($\alpha = 0.13$) and Pb-NBSA ($\alpha = 0.20$). The method employed by Tanford has been discussed fully in the Introduction (see p. 16). For reasons which will become

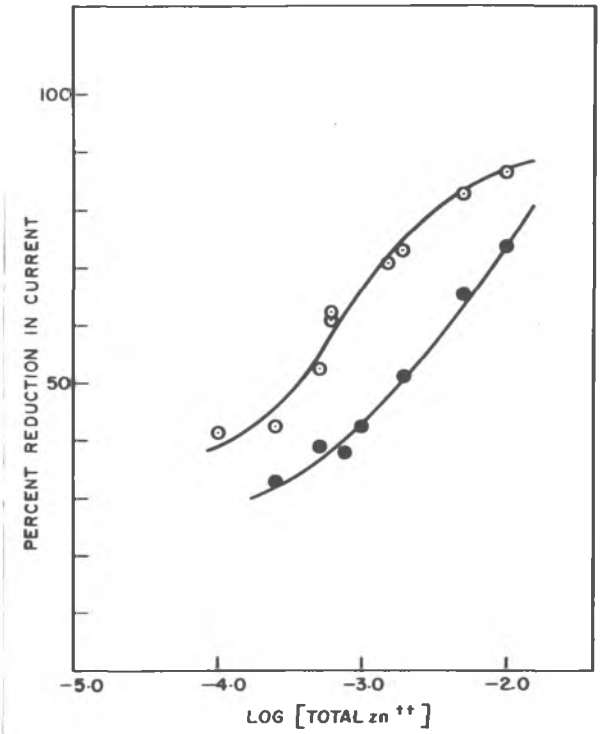


Fig.23 : Effect of the Albumins on the diffusion current of Zn^{++} ions (acetate buffer pH 6.5 & μ = 0.20; 30°C).

—●— ABSA

—⊙— NBSA

obvious later we have preferred to obtain an absolute value of α under the experimental conditions used. A single equilibrium dialysis run will normally suffice for the purpose. From the diffusion currents given by the solutions on either side of the dialysis bag, α may be evaluated. Thus $\alpha = \frac{i_d(\text{internal}) - i_d(\text{external})}{C' r \cdot Cp}$ where i_d(internal) and i_d(external) are the diffusion currents given by the equilibrium-dialysed solutions and C, r & Cp are as defined in Equations 19 and 24. It may be noticed that r may be evaluated from the equilibrium-dialysis experiment itself.

Experimental

The standard polarographic procedure detailed previously (see chapter II) was followed. Measurements were made in the standard acetate buffer solution which acted as the supporting electrolyte. The protein concentration was kept constant at 1.0%. Nitrogen was bubbled through the solutions for 30-45 minutes to remove dissolved oxygen. The solutions were then transferred to the polarographic cell for the measurement of diffusion current. No maximum suppressor was required for these solutions. The constant α was evaluated from separately conducted equilibrium-dialysis experiments. All measurements were made at 30 $\pm$ 0.1°C.

Results and Discussion

The values of $\frac{(i_d)_p}{i_d}$ are represented in Fig.23 as a function of the logarithm of the total metal ion concentration (A_o), for the system Zn-NBSA and Zn-ABSA. Similar plots for

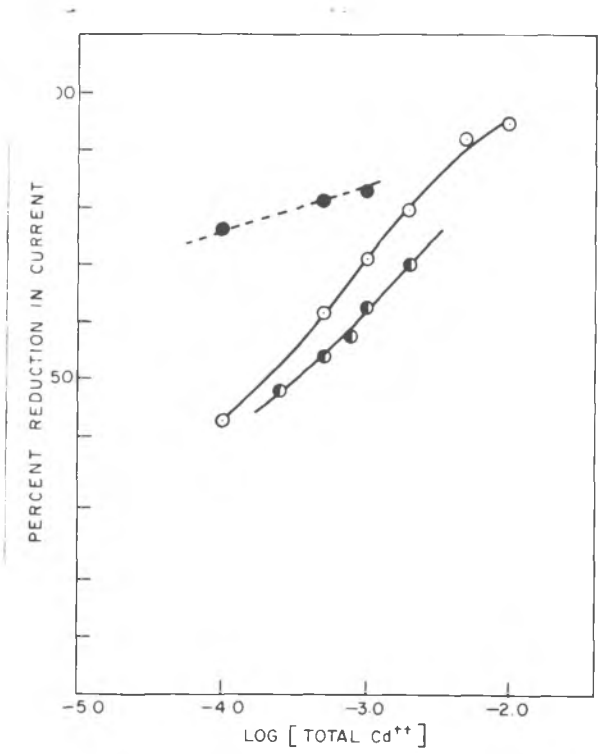


Fig.24 : Effect of the Albumins on the diffusion current of Cd^{++} ions (acetate buffer pH 6.5 & μ = 0.20; 30°C).

—◐— ABSA;

—⊙— NBSA;

—●— EBSA.

the cadmium-serum albumin systems are shown in Fig.24. It may be noted that the acetylated albumin causes a much larger reduction in the diffusion current than the native protein due, no doubt, to the greater binding of the metal ions by the acetylated derivative. The esterified derivative does not seem to have much ability to bind the cadmium ion - a conclusion supported by equilibrium-dialysis experiments described in chapter II. Similar experiments for the system Zn-EBSA, however, were not successful as the ester shifted the potential for hydrogen-ion discharge to the vicinity of that for the zinc ion, rendering the polarographic estimation of the requisite diffusion current impossible. It has, however, been shown earlier that the esterified derivative has nearly as much binding power for the zinc ion as the native protein (Chapter II).

The constant α was determined and found to be independent of the metal ion concentration over the range 1.0×10^{-4}M - 2.0×10^{-3}M. Measurements at larger concentrations of the metal ion were, however, less reliable . The relevant values of α for the systems studied are shown in Table VIII.

Table - VIII

The values of α in an acetate buffer (pH 6.5 and ionic strength 0.20) at 30°C.

	Zn^{++}	Cd^{++}
NBSA	0.22 ± 0.03	0.30 ± 0.02
ABSA	0.20 ± 0.03	0.28 ± 0.01

With the values of $\frac{(i_d)_p}{i_d}$ (Figs.23 & 24) and of the constant α (Table VIII) it is easy to evaluate the binding data. Good agreement is indicated with the binding data obtained from equilibrium-dialysis (See Fig.8, 10 & 11). It may be remarked that such an agreement between the polarographic and equilibrium-dialysis data is only to be expected in view of the fact that the constant α has been evaluated from equilibrium-dialysis experiments. This drawback does not, however, vitiate the suitability of the polarographic method in that a single equilibrium-dialysis experiment is often sufficient for an evaluation of α. Furthermore, the binding data calculated from the polarographic measurements are relatively insensitive to appreciable errors in the value of α. It may also be pointed out that polarographic measurements may conveniently be employed to extend the binding data towards larger metal ion concentrations where equilibrium-dialysis experiments are both tedious and unreliable requiring frequent repetitions. With the added advantage of quick measurements, the polarographic method has everything to recommend itself wherever it can be used.

It may be of interest to speculate on the nature of the constant α. Saroff and Mark[26] had equated this constant to the square root of the ratio of the diffusion coefficients of the protein and the metal ion; thus

$$\alpha = \left[\frac{D \text{ albumin}}{D \text{ metal ion}}\right]^{\frac{1}{2}}$$

From the data of Champagne[55] the diffusion coefficient of

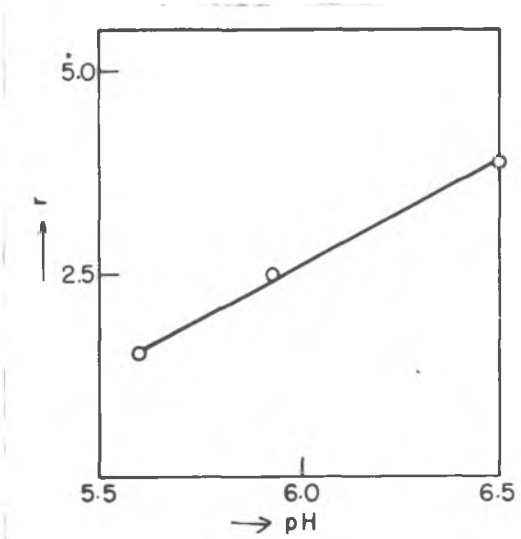


Fig.25 : Effect of pH on the binding of Zn^{++} ions by NBSA. (acetate buffers of μ = 0.20; total Zn^{++} ion concn 1.0×10^{-3} M; Protein concn 1%).

NBSA at pH 6.5 is 6.0×10^{-7} cm^2/sec at 20°C. The polarographic diffusion coefficient of the zinc and the cadmium ion is 0.65×10^{-5} cm^2/sec at 20°C [23]. Thus, the value of α should be of the order of 0.30 which compares favourably with the experimental values of 0.22 for zinc and of 0.30 for cadmium (see Table VIII). It would thus appear that the contribution of the metal protein ion to the diffusion current is governed largely by the diffusion coefficients of the protein and of the metal ion.

That the above is not a completely true picture of the nature of α is revealed by the fact that its value is affected by pH in a manner contrary to what should be expected from a consideration of the diffusion coefficients alone. A few experiments were conducted to study the effect of pH on this constant and on the binding of zinc ions by NBSA. The binding studies were made at pH 5.60, pH 5.93 and pH 6.50 in an acetate buffer of ionic strength 0.20. Keeping the protein concentration fixed at 1% and the zinc ion concentration at 1.0×10^{-3} M, the extent of the binding and the value of α were determined by equilibrium dialysis supplemented by polarographic measurements. The binding data are plotted in Fig. 25; the values of α are given in Table IX.

Table - IX

The effect of pH on the value of constant α for the systems Zn^{++}-NBSA & Cd^{++}-NBSA

pH	Zn^{++}	Cd^{++}
5.60	0.37	-
5.93	0.35	-
6.50	0.22	0.30
limiting value (Tanford)	0.10	0.18

Champagne[55] has reported that in the pH range 3.95 - 7.40, the diffusion coefficient of NBSA increases slightly with pH. This should result in a slight increase in the value of α with an increase in pH, a conclusion which is contrary to our observations. That the value of α does, infact, decrease with pH is supported by the fact that the values reported by Tanford are appreciably less - a result which may be expected from an extension of our data (Table IX) to pH values higher than pH 6.5. It would thus appear that the suitability of the limiting method of Tanford[24] for the evaluation of α needs to be reconsidered for the simple reason that this constant is pH-dependent.

It is obvious, therefore, that whereas diffusion may play a predominant role in determining the value of α , there may be other factors involved which govern its pH - dependence. It is difficult, in the present state of our knowledge, to comment on these modifying factors.

We shall now try to evaluate the intrinsic association constants for the binding of zinc and cadmium ions by serum albumins. It is easy to calculate from equation 21, the value of $\frac{(i_d)_p}{i_d}$ for a system in which half the metal is free and the other half protein-bound. For a general system in which the fraction of the metal ion bound is x, the value of $\frac{(i_d)_p}{i_d}$ may be obtained from the equation :

$$\left[\frac{(i_d)_p}{i_d}\right]_x = (1 - x) + x\alpha \quad \ldots\ldots \quad (27)$$

From the value of $\frac{(i_d)_p}{i_d}$ thus calculated it is possible to

read off the corresponding total metal concentration, A_o , from the corresponding $\frac{(i_d)_p}{i_d}$ vs A_o curves. The free metal ion concentration, A, will thus be A_o (1- x). The number of metal ions, r, bound per mole protein will be $\frac{xA_o}{Cp}$

We may now proceed to discuss the metal serum albumin interaction in terms of a competition between the hydrogen and the metal ion for combining with the eighteen imidazole groups of protein molecule. Thus,

$$\frac{r}{(n_t - r - n_{HIm})A} = k^o_{MIm} e^{-2wZ_pZ_M} \quad \ldots\ldots (22)$$

and

$$\frac{n_{HIm}}{(n_t - r - n_{HIm})a_H} = k^o_{HIm} e^{-2wZ_pZ_H} \quad \ldots\ldots (23)$$

where the various terms are as described previously (see p. 17).

For an evaluation of k^o_{MIm} the intrinsic association constant for the interaction of the metal ion with the imidazole groups of the albumin molecule, values of r_{HIm} at the corresponding values of r must be known. The values of the other terms in eqn.22 are obtained on the assumption that the metal uptake involves a singly charged $(MAc)^{+1}$ complex ion. This is a reasonable assumption in view of the similar postulates made by earlier workers. We then have a value of 0.03 for w as calculated from the Debye-Huckel theory (eqn.18). Zp may simply be defined as $Z_p = Z^o_\rho + r_m$ where Z^o_ρ is the charge on the protein, assumed to have a value of -16 for NBSA and -39 for ABSA (see Chapter I), k^o_{HIm} has a log k^o_{HIm} value of 6.10 (Table V). It is thus possible to evaluate n_{HIm} from eqn.23

Table X-A

Values of log k^o at different fractions of the metal bound by the protein

Fraction bound of total metal	Zinc							
	NBSA				ABSA			
	$\frac{(i_d)_p}{i_d}$	A_o	r	log k^o	$\frac{(i_d)_p}{i_d}$	A_o	r	log k^o
80 %	-	-	-	-	0.360	4.57×10^{-4} M	2.52	2.96
75 %	0.415	1.51×10^{-4} M	0.78	2.98	0.400	7.24×10^{-4} M	3.75	2.88
66.7%	0.480	3.16×10^{-4} M	1.46	2.84	0.467	1.38×10^{-3} M	6.35	2.80
60 %	-	-	-	-	0.520	2.04×10^{-3} M	8.45	2.78
50 %	0.610	6.92×10^{-4} M	2.36	2.56	-	-	-	-
33.3%	0.740	1.78×10^{-3} M	4.09	2.34	-	-	-	-
25 %	0.805	3.55×10^{-3} M	6.14	2.27	-	-	-	-
20 %	0.844	6.31×10^{-3} M	8.71	2.29	-	-	-	-

Table X-B

Values of log k^o at different fractions of the metal bound by the protein

Fraction bound of total metal	Cadmium							
	NBSA				ABSA			
	$\frac{(i_d)_p}{i_d}$	A_o	r	log k^o	$\frac{(i_d)_p}{i_d}$	A_o	r	log k^o
80 %	0.475	1.74×10^{-4} M	0.91	2.99	0.46	2.0×10^{-4} M	1.04	2.79
66.7%	0.533	2.88×10^{-4} M	1.32	2.88	0.520	4.27×10^{-4} M	1.97	2.64
60 %	-	-	-	-	-	-	-	-
50 %	0.650	6.92×10^{-4} M	2.36	2.56	0.640	1.32×10^{-3} M	4.55	2.43
33.3%	0.767	1.59×10^{-3} M	3.66	2.22	0.76	3.55×10^{-3} M	8.1	2.28
25 %	0.825	2.34×10^{-3} M	4.09	2.17	-	-	-	-
20 %	-	-	-	-	-	-	-	-

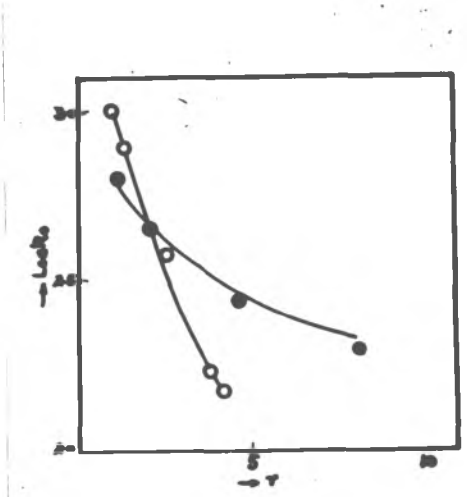


Fig.26 A : Effect of Cd^{++} ion uptake on the intrinsic association constants for interaction with the albumins (acetate buffer pH 6.5 & μ = 0.20).

—⊙— NBSA;

—●— ABSA.

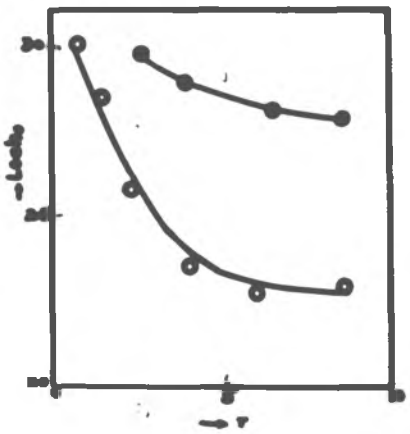


Fig.26 B : Effect of Zn^{++} ion uptake on the intrinsic association constants for interaction with the albumins. (acetate buffer pH 6.5 and μ = 0.20).

—⊙— NBSA;

—●— ABSA.

and eventually the intrinsic association constant k^{o}_{MIm} from eqn.22.

The detailed calculations together with the values of the intrinsic association constant finally obtained, for various fractions of the total metal ions bound by NBSA and ABSA are given in Table X-A & B. The log k^{o}_{MIm} vs r graphs are shown in Figs.26-A & B. It is obvious from Figs.26A & B that k^{o}_{MIm} for any given system, decreases with the number of metal ions bound by the protein. This decrease is much more rapid for the metal - NBSA than for the metal - ABSA systems. A comparison of the curves given in Fig.26-A with the corresponding curves of Fig.26B further reveals that the k^{o}_{MIm} decreases much more rapidly for the systems involving the cadmium ion than for those involving the zinc ion.

Tanford has reported that the values of k^{o}_{MIm} for the binding of the zinc and cadmium ions by the imidazole groups of serum albumin remain practically unaltered at 25%, 50% and 75% combination[24]. As the systems investigated by Tanford covered only a narrow range (0.6-1.7) of r, it is likely that the effect of r on the value of k^{o}_{MIm} may not have been noticed. A decrease in k^{o}_{MIm} for the system, Cu-NBSA, was however, observed by Tanford.

It is reasonable to expect that if the metal ion is bound by the imidazole groups of the albumin molecule alone and further if all the imidazole groups are equivalent, the value of the intrinsic association constant, k^{o}_{MIm} should not be

influenced by the amount of metal ions taken up. In view of the results described above, it is probable that either or both of the following may be operative in reducing the value of k^{o}_{MIm} calculated on the basis of the imidazole groups being the reactive sites.

(i) All the eighteen imidazole groups may not be equivalent

(ii) The contribution of competing functional groups, possibly the carboxyl groups of the protein molecule, may become pronounced as more and more metal ions are taken up by the protein.

An extrapolation of the curves of Fig.26-A & B to $r = 0$ leads to the conclusion that the intrinsic association constant of the cadmium-albumin and zinc-albumin systems has a log k^{o}_{MIm} value of approximately 3.1. This is only a little larger than the values reported for the first association constant of imidazole with zinc and cadmium ions (see Table V). It is, therefore, reasonable to conclude that the initial stages of the binding process involve the interaction of the cadmium, zinc (and copper) ions at the imidazole sites of the albumin molecute. As the number of bound metal ions increases, however, the contribution of the imidazole groups to the interaction process may be reduced for reasons enumerated above.

-o-o-o-

: C H A P T E R - IV :

ELECTROPHORETIC MOBILITY STUDIES

(Acetate buffer, pH 6.5, ionic strength 0.20)

It has already been pointed out (see p.40) that the electrophoretic mobility method has been used to follow complex formation between proteins and anions, fatty acids, detergents etc. and also that this method does not appear to have been used for a study of metal protein interactions. It is our object in this chapter to investigate the effect of the binding of copper, zinc, cadmium and cobalt ions on the electrophoretic mobility of bovine serum albumin and its derivatives.

Experimental

The mobility measurements were made in the standard acetate buffer. 1% protein solutions were used. Complexes of the metal with the protein were prepared by adding suitable amounts of the metal - calculated from the binding data - to the protein solution. Requisite metal additions corresponding to the free metal ion concentration in equilibrium with the metal-protein complex were made to the buffer solution. The boundaries were formed between these two solutions. The solutions on either side of the boundary thus correspond to those obtaining in the equilibrium dialysis with a possible difference in that the buffer concentration was identical in both solutions.

The mobility measurements were made in a Tiselius Electrophoresis Apparatus (Perkin-Elmer model 38)[56] using the scanning system devised by Longsworth[57]. A 2 ml. cell was used. The experiments were conducted at 0.5°C. The standard procedure for the formation of boundaries was employed.[56] After thermostating the cell assembly in the bath for 2-3 hours, the boundaries were brought into view with an automatic compensating device and their initial positions photographed. The positions of the boundaries were also photographed at regular intervals during the electrophoresis run. The runs were usually made for 1-3 hours depending on the nature of the protein. Mobilities were calculated from the displacements in the descending boundary. Only those runs in which the displacement of the boundary was a linear function of time (at constant current) were used for the evaluation of mobilities.

The evaluation of mobility involved an accurate measurement of the current passing through the cell. For this purpose a standard resistance was inserted in the circuit in series with the cell; the potential difference across the standard resistance was measured accurately with a potentiometer. The current passing through the cell could thus be measured to $\pm$ 0.1%. The mobility may be evaluated from the simple relation

$$\mu = \frac{d\ A\ K}{it}$$

where μ is the mobility in cm^2/volt/sec, d the boundary

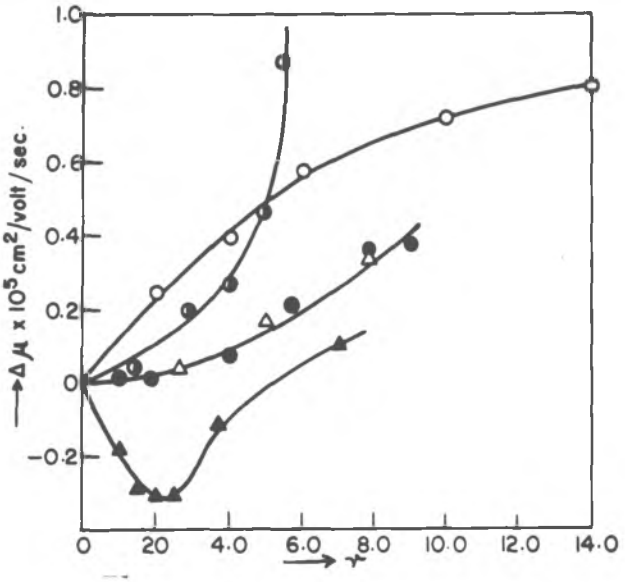


Fig.27 : Effect of the binding of metal ions on the electrophoretic mobility of NBSA (acetate buffer pH 6.5 & μ = 0.20; 0°C).

- —⊙— Co^{++} (Cobalt chloride);
- —◐— Cd^{++} (Cadmium acetate);
- —●— Zn^{++} (Zinc sulphate);
- —△— Zn^{++} (Zinc acetate);
- —▲— Cu^{++} (Copper acetate);.

displacement in cms, t the time in secs, A the cross-sectional area of the cell (0.3 cm^2), K the specific conductance of the protein solution and i the current in amperes. The conductivities of the protein solutions were measured at $0^{\circ}C$ with a Leeds & Northrup Conductivity Bridge using a conductivity cell with a cell constant of 12.6. The conductivity cell had a capacity of about 0.5 ml.

The boundary displacements were measured from the schlieren patterns with a travelling microscope. In the systems involving inhomogeneous proteins, such as the acetylated B.S.A., the schlieren patterns were enlarged and the boundary displacements measured from the first moment of the entire gradient curve.

Analar cupric chloride and cupric acetate, Merck zinc sulphate, zinc acetate, cadmium acetate and cobalt chloride were used as the source of metal ions in these studies.

Results and Discussion

The electrophoretic behaviour of bovine serum albumin and its esterified and acetylated derivatives has already been described in Chapter I.

Metal - NBSA System : The mobility increment, $\Delta\mu(\mu_{PM_i} - \mu_P)$ is plotted in Fig.27 as a function of r, the number of metal ions bound per protein molecules. It may be noticed from Fig.27 that the slope of the $\Delta\mu$ vs r curves, at least for the first few metal ions bound is positive for the system Co-NBSA and changes in the order Co-NBSA > Cd-NBSA > Zn-NBSA. The

corresponding curve for the system Cu-NBSA, however, shows a negative slope - a result which is contrary to what should be expected from the uptake of a metal ion by the protein. The values of the slopes, necessarily of a relative nature in view of the shapes of $\Delta\mu$ vs r curves, are given in Table XI for the initial stages of the binding process. As the metal uptake increases, however, the mobility of the protein is affected in a profound manner by the nature of the metal ion bound. Thus the mobility of serum albumin tends towards a steady value as more and more cobalt ions are bound. This is in contrast to the behaviour of Cd-NBSA and Zn-NBSA systems wherein the mobility increases more and more as the value of r is increased, the effect being more pronounced in the former than in the latter. The mobility increment for copper - NBSA system becomes zero at an r value of about 2 and then becomes positive as more copper ions are bound.

Table - XI

The effect of metal-uptake on the mobility of bovine serum albumins.
(acetate buffer, pH 6.5, ionic strength 0.20)
The data refer to the first few metal ions bound

Bound Metal	NBSA	ABSA	EBSA
Cobalt	0.10	0.28	--
Cadmium	0.05	0.17	--
Zinc	0	0	0
Copper	-0.17	0	0

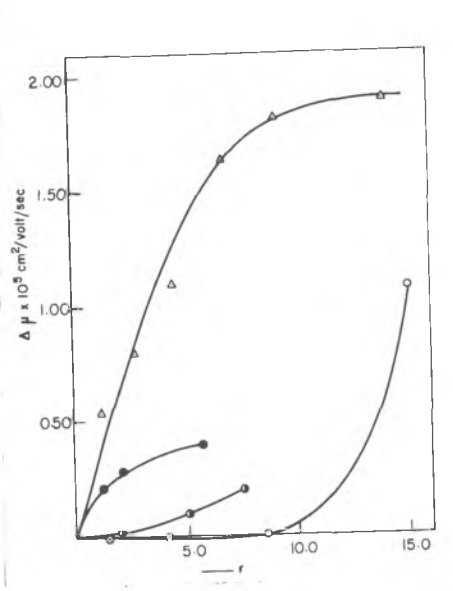


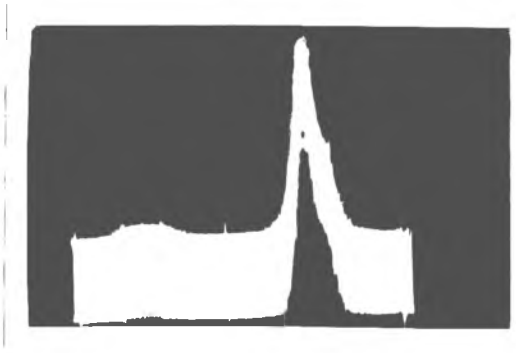
Fig.28 : Effect of the binding of metal ions on the electrophoretic mobility of ABSA (acetate buffer pH 6.5 & μ = 0.20; 0°C).

—△— Co^{++} (Cobalt chloride)

—●— Cd^{++} (Cadmium Acetate)

—◑— Cu^{++} (Copper chloride)

—⊙— Zn^{++} (Zinc Sulphate)

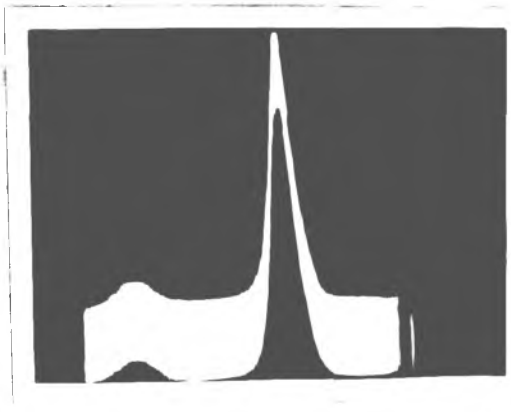


(A)

Fig.29 : Effect of the binding of Zn^{++} ions on the electrophoretic homogeneity of ABSA (acetate buffer pH 6.5 & μ = 0.20; 0°C). (Period of electrophoresis = 75 mins; field strength = 1.3 volts).per cm.).

(A) r = 9

(B) r = 15.



(B)

(A)

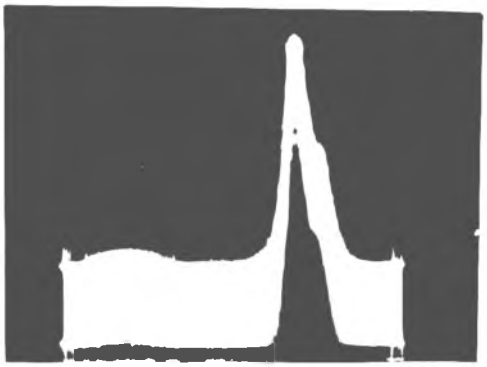
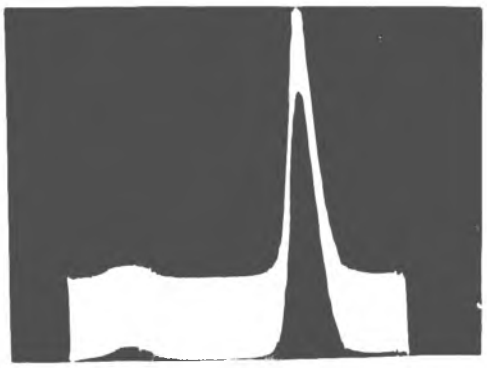


Fig.30 : Effect of the binding of Cu^{++} ions on the electrophoretic homogeneity of ABSA (acetate buffer pH 6.5 & μ = 0.20) 0°C). (Period of electrophoresis = 75 mins; field strength = 2.0 volts per cm.).

(A) r = 2;

(B) r = 8.

(B)



Metal - ABSA Systems : The $\Delta\mu$ vs r curves for the metal-ABSA systems are shown in Fig.28. The slopes of the curves in the initial stages of the binding process are given in Table XI. It may be seen that the mobility increment is relatively much more for ABSA than for NBSA in so far as the uptake of cobalt and cadmium ions is concerned. For copper - ABSA and Zn - ABSA systems, the mobility is affected only slightly. As more and more cobalt ions are bound the mobility of ABSA tends towards a steady state. The Cd - ABSA systems behaves likewise, - an observation which is in contrast to the electrophoretic behaviour of the system Cd - NBSA. The systems copper - ABSA and zinc - ABSA differ markedly from the corresponding systems involving NBSA (compare Figs 27 & 28).

In the course of the electrophoretic investigation of the metal - ABSA systems, it was observed that whereas the uptake of cobalt and cadmium ions did not affect the shape of the schlieren pattern of the protein, the uptake of copper and zinc ions tended to make the protein electrophoretically homogeneous. The phenomena are illustrated in Fig.29 A & B for zinc - ABSA and Fig.30 A & B for the copper - ABSA systems. It is obvious from Figs 29 and 30 that the protein becomes electrophoretically homogeneous with the uptake of about 8 copper ions and of about 15 zinc ions.

Metal - EBSA Systems : The effect of the binding of zinc and copper ions by the esterified albumin on its mobility is illustrated in Fig.31. As the protein has a high positive charge it is

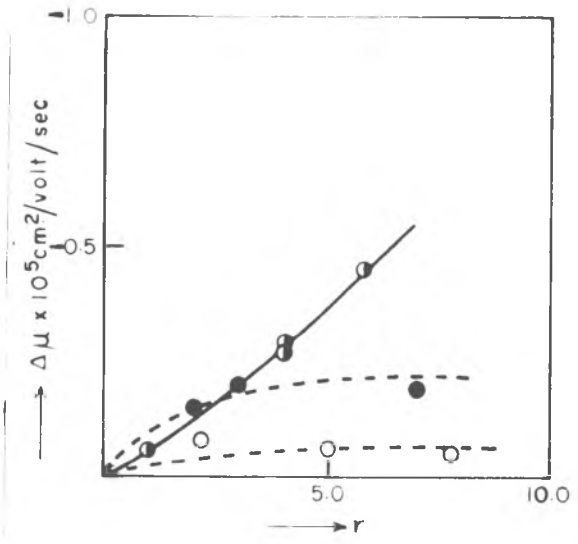


Fig.31 : Effect of the binding of metal ions on the electrophoretic mobility of EBSA (acetate buffer pH 6.5 & μ = 0.20; 0°C).

—⊙— Zn^{++} (Zinc acetate);

—◑— Zn^{++} (Zinc sulphate);

—⊙— Cu^{++} (Copper acetate).

imperative that the metal be added only as metal acetate. Thus the addition of $ZnSo_4$ caused considerable decrease in the mobility of the protein indicating that the sulphate ions are bound by the protein. The addition of zinc as zinc acetate caused only a slight reduction in the mobility. The binding data obtained with zinc sulphate are, however, identical with those obtained with zinc acetate (see Fig.10). It may be noticed from Fig.31 that the mobility of EBSA is only slightly reduced by the uptake of copper and zinc ions. Cadmium and cobalt are bound little by this protein and, as such, have no appreciable effect on its mobility.

The above results suggest that the electrophoretic behaviour of metal - protein systems is rather complex. If the binding process were to involve the simple uptake of a metal ion (possibly MAc^+ ion) the electrophoretic behaviour of all the systems studied should have been identical i.e., the mobility of the metal protein complex should have been a linear function of the number of metal ions bound.

The systems investigated by us may be classified, at least for the first few metal ions bound, as follows :

(1) Those which show a linear increase in mobility with the number of metal ions bound. Co-NBSA and Co-ABSA.

(2) Those which show either a slight decrease or none at all in the mobility. Cu-EBSA, Cu-NBSA (Cu-ABSA), Zn-EBSA, Zn-ABSA.

(3) Those which are intermediate between (1) and (2). Zn-NBSA, Cd-NBSA and Cd-ABSA.

It seems to us that the above results may probably be explained on the basis of the effect of the metal uptake on the dissociation of the various functional groups of the protein molecule and on the binding of buffer anions. It is reasonable to expect that an increase in the positive charge on the protein consequent on metal uptake will decrease the affinity of the functional groups for the hydrogen ion and to increase the uptake of the buffer anion. Taking these factors into account the mobility-behaviour of the metal-albumins may, as will be shown later, be explained satisfactorily.

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G E N E R A L D I S C U S S I O N

It is our object to discuss the interaction of bovine serum albumins with metal ions in terms of a competition between the metal and hydrogen ions for the same sites of the protein molecule. We shall assume that the guanidinium, sulfhydral, the phenolic, α -and ϵ - amino groups, in view of their high pK^{o} values, are present in the acid form at pH 6.5 and are not available for binding the metal ion. We shall, therefore, deal with equilibria involving hydrogen and metal ions on the one hand and the imidazole and carboxyl groups of the protein molecule on the other.

1. Equilibria in Serum Albumin Solution :

The dissociation of the functional groups in a given set of sites may be simply represented as[5]

$$\log \frac{n_B}{n_t - n_B} = pH - pK^{o} - \frac{2wZ_p}{2.303} \quad \ldots . \quad (28)$$

n_t is the total number of a given type of functional groups, n_B the number in the basic form and w & Z_p are as described previously (see p.17). It follows from eqn.28 that 105 carboxyl groups of NBSA are dissociated and 103 of the acetylated derivative. In the esterified derivative all the hundred and six carboxyl groups are blocked, and are not available for reaction with the proton.

An application of eqn.28 to the imidazole groups of the albumins reveals that, of the eighteen groups, 16.9

Table XII

Charge balance in serum albumin solutions
(acetate buffer pH 6.5 and ionic strength 0.20)

	number of Guanidinium groups	number of amino groups	number of Imidazolium groups	1+2+3	number of Carboxyl group	(4)-(5)	Z_p^o	number of Ac^- ions bound 6-7	$k_{Ac^-}^o$
EBSA	23	63	1.1	87.1	0	+87	+30	57	1.52
NBSA	23	63	9.2	95.2	105.1	-10	-16	6	0.88
ABSA	23	11	14.5	48.5	102.6	-54	-33	(-15)	-
NBSA (at pH 4.7)	23	63	16	102	88	+14	0	+14	-

exist in the neutral (basic) form in the esterified derivative, 8.8 in the native serum albumin and only 3.5 in the acetylated derivative at pH 6.5.

2. Charge balance in protein solutions :

The charge balance in the albumin solutions in the acetate buffer of pH 6.5 and ionic strength 0.20 is given in Table XII. Assuming that the buffer anion is not bound by the protein, the net charge should be + 37, - 10 and - 54 for EBSA, NBSA and ABSA respectively at pH 6.5. These values (column 6. Table XII) differ markedly from those evaluated from mobility measurements. It is reasonable to assume, therefore, that the acetate ion is bound by the protein to the extent of 57 and 6 acetate ions per mole of EBSA and NBSA respectively (column 8 Table XII).

3. The binding of buffer anions :

The interaction of proteins with anions has been shown to involve the charged cationic groups of the proteins[41]. The equilibrium may be represented as,

$$\frac{n_{A\bar{C}}}{(n_c - n_{A\bar{C}})a_{A\bar{C}}} = k^o_{A\bar{C}} \exp(2wZ^o_p) \quad \ldots\ldots \quad (29)$$

where n_c is the total number of cationic groups given in column 4 Table XII, $n_{A\bar{C}}$ the number of acetate ions bound, $a_{A\bar{C}}$, the activity of free acetate ions at equilibrium, $k^o_{A\bar{C}}$, the intrinsic association constant for the binding of acetate ions and Z^o_p the charge on the protein as given in column 7

Table XII. With the values of n_{Ac^-} as given in Table XII, it is thus possible to evaluate the intrinsic constant $k^o_{Ac^-}$ from eqn.29. These values are given in column 3 Table XII.

No quantitative work on the binding of the acetate ion by serum albumin has been reported. It is, however, of interest to examine the results obtained by Longsworth and Jacobsen[32]. These authors found that the isoelectric pH of bovine serum albumin in 0.1 N acetate was 4.71. Assuming the isoionic pH of bovine serum albumin to be 4.90[58], we have, following Scatchard[58].

$$n_{Ac^-} = \frac{2.303}{2w} \cdot \Delta \text{ pH} \quad \ldots\ldots (30)$$

With a value of 0.19 for Δ pH and 0.03 for w, the number of acetate ions bound at the isoelectric pH in 0.1 N acetate solution is 7.3. From the charge balance at pH 4.71, the number of acetate ions bound comes to 14 (Table XII). The value of $k^o_{Ac^-}$ as calculated from eqn.29 will, then, be 0.76 on the basis of 7.3 bound acetate ions and 1.57 on the basis of 14 acetate ions bound per mole of the protein. These values compare favourably with those reported in column 3, Table XII.

In what follows we have assumed a value of 0.88 for the intrinsic association constant of the binding of acetate ions by bovine serum albumin and 1.52 for the binding by the esterified derivative. As we are interested mainly in the change in the binding of acetate ions consequent on the uptake of metal ions, the dual value used for $k^o_{Ac^-}$ may not be particularly objectionable.

The ability of the acetylated derivatives to bind the

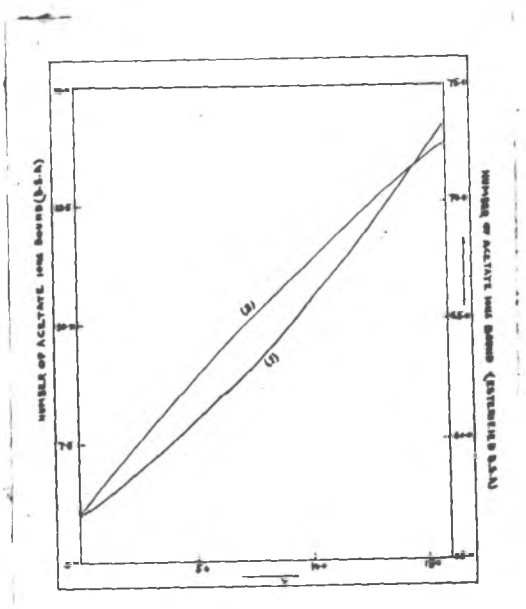

Fig.32 : Effect of metal uptake on the binding of acetate ions by the albumins (acetate buffer pH 6.5 & μ = 0.20).

(1)- NBSA;

(2)- EBSA.

acetate ion may be shown to be practically nil. We are thus led to expect this protein to have a charge of -54 as against the value of -39 obtained from mobility measurements. The binding of acetate ions will tend to make the discrepancy even larger. This protein is, however, electrophoretically inhomogeneous for reasons which are not understood at present. The charge on the acetylated derivative has, therefore, been assumed to be -39.

4. Equilibria in metal-protein systems :

The equilibrium conditions in the solutions of serum albumin and its derivatives in the acetate buffer of pH 6.5 and ionic strength 0.20 have been fully discussed above. We shall now examine how the various equilibria are influenced by the uptake of metal ions. A tacit assumption has been made in that the metal uptake is assumed to involve the singly charged MAc^+ complex ion. The binding of 'r' metal ions will then increase the charge on the protein by 'r' units. This will tend to increase the binding of acetate ions by the protein and to decrease the binding of hydrogen ions by the functional groups of the protein.

The effect of metal-uptake on the binding of acetate ions by the serum albumins may be evaluated from eqn.29. The results are plotted in Fig.32. It may be noticed that the uptake of metal ion results in a considerable increase in the number of acetate ions bound by the protein, the effect being more pronounced for the esterified than for the native albumin.

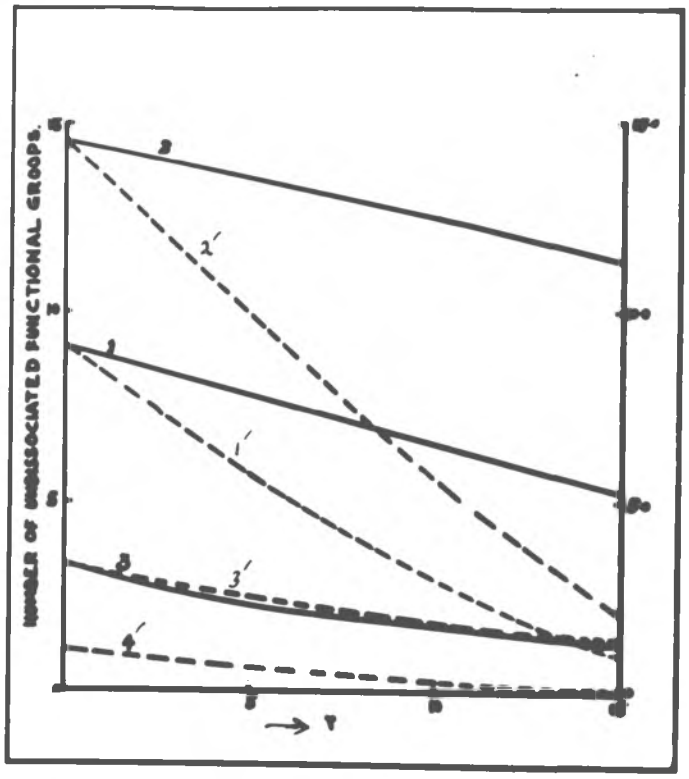

Fig.33 : Effect of metal uptake on the dissociation of functional groups of the albumins (acetate buffer pH 6.5 & μ = 0.20).
Full curves refer to the binding at the carboxyl groups and dashed curves to the binding at the imidazole groups.

1 NBSA imidazole groups;
2 ABSA imidazole groups;
3 ABSA carboxyl groups;
4′ EBSA imidazole groups.

The effect of metal binding on the dissociation of the imidazolium and carboxyl groups will depend on the functional group with which the metal ion reacts. If the combination takes place with the basic imidazole groups, we have,

$$\frac{n_{HIm}}{(18-r-n_{HIm})(H^+)} = k^o_{HIm}\, e^{-2wZ_p} \quad \ldots\ldots \quad (31)$$

and

$$\frac{n_{C\bar{O}\bar{O}H}}{(106-n_{C\bar{O}\bar{O}H})(H^+)} = k^o_{C\bar{O}\bar{O}H}\, e^{-2wZ_p} \quad \ldots\ldots \quad (32)$$

If, however, the metal-uptake occurs at the carboxyl groups of the protein, we have,

$$\frac{n_{HIm}}{(18-n_{HIm})(H^+)} = k^o_{HIm}\, e^{-2wZ_p} \quad \ldots\ldots \quad (33)$$

and

$$\frac{n_{C\bar{O}\bar{O}H}}{(106-r-n_{C\bar{O}\bar{O}H})(H^+)} = k^o_{C\bar{O}\bar{O}H}\, e^{-2wZ_p} \quad \ldots\ldots \quad (34)$$

In the above equations $Z_p = Z^o_p + r$, $w = 0.03$, k^o_{HIm} and $k^o_{C\bar{O}\bar{O}H}$ are the intrinsic association constants of the imidazole and carboxyl groups, respectively, with the proton (see Table III).

The number of the undissociated (acid) functional groups are plotted in Fig.33 as a function of the number of metal ions bound by the protein at pH 6.5. The full curves refer to the binding of the metal ions at the carboxyl groups,

groups of the

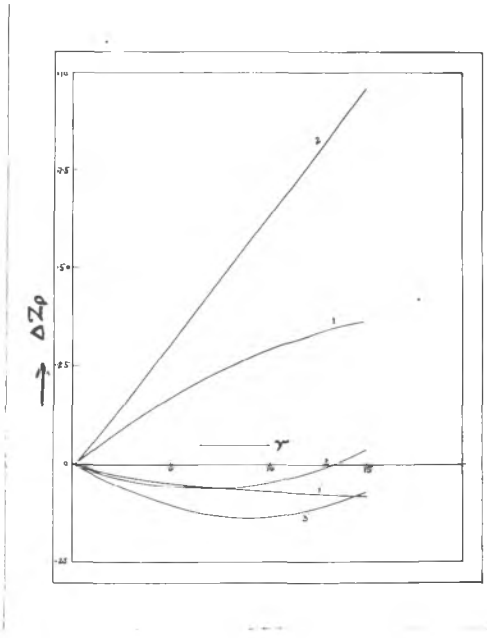

Fig.34 : Effect of metal uptake on the net charge on the serum albumins (acetate buffer pH 6.5 & μ = 0.20).

1 NBSA (Carboxyl binding);
2 ABSA (Carboxyl binding);
3' EBSA (imidazole binding).

1' NBSA (imidazole binding).
2' ABSA (imidazole binding).

protein. It may be noticed (Fig.33) that the dissociation of the functional groups is favoured by the uptake of metal ions by the protein.

5. Charge balance in Metal-protein systems :

We are now in a position to study the effect of the binding of metal ions on the net charge on the protein. The net increase in charge may be represented as

$$\Delta Z_p = r - \Delta n_{Ac^-} + \Delta n_{HIm} + \Delta n_{COOH} \quad . \quad . \quad . \quad . \quad (35)$$

The net change in the charge on the protein is plotted in Fig.34 as a function of the number of metal ions bound. It may be immediately noticed from Fig.34 that the interaction of the imidazole groups of the protein with the metal ions should result in a slight decrease in the net charge of the protein. If, however, the binding process involves the carboxyl groups of the protein, the net charge on the proteins should increase, the effect being nearly twice as much for the system M-ABSA than for the system M-NBSA.

A comparison of $\Delta\mu$ vs r curves for the Cobalt-NBSA and Cobalt-ABSA systems (Figs 27 & 28) with the curves in 1 & 2 of Fig.34 suggest that the binding of the cobalt ion occurs essentially at the carboxyl groups of the albumin molecule. The $\Delta\mu$ vs r curves for copper-EBSA and zinc-EBSA fit almost quantitatively with the corresponding calculated curve (Curve 3 Fig.34). The $\Delta\mu$ vs r curves for copper-NBSA, zinc-NBSA

and cadmium-NBSA, copper-ABSA, zinc-ABSA and cadmium-ABSA seem to be characterized by a relatively strong binding with the imidazole groups at low binding, with a pronounced participation of the carboxyl groups of the protein as more and more metal ions are bound. The effect of the carboxyl groups seems to be more for the systems involving the cadmium ion than for those involving the zinc and copper ions. The mobility measurements thus give further support to the conclusions previously arrived at from equilibrium dialysis and polarographic studies (see Chapters II and III).

6. Intrinsic association constants :

It has been shown above that the mobility data can be satisfactorily explained in terms of a competition between the hydrogen and metal ions for the sites on the protein molecule. We may now proceed to analyse the binding data on the basis of the above concepts. An examination of Fig.33 reveals that the number of the dissociated imidazole groups increases as the number of metal ions bound at these sites is increased. To a first approximation we may write

$$(18 - n_{HIm}) = n_o + \beta r \, , \quad \ldots\ldots \quad (36)$$

where $(18 - n_{HIm})$ is the number of imidazole groups not covered by the proton, n_o the number of dissociated imidazole groups at $r = 0$ and β has a value of approximately 0.9 for ABSA, 0.7 for NBSA and 0.1 for EBSA (Fig.33).

The term n in the statistical equation should therefore, be replaced by (18 - HIm). We, then, have

$$\frac{r}{A} = kn_o - (1 - \beta)kr \quad \ldots\ldots\ldots \quad (37)$$

A plot of $\frac{r}{A}$ vs r (in the absence of electrostatic interaction) should, therefore, give a straight line with the intercepts kn_o on the Y - axis and $\frac{n}{1-\beta}$ on the X - axis. It is apparent that the number of imidazole groups available for reaction with the first metal ion is only a fraction of the total, the values being 3.5, 8.8 and 16.9 for ABSA, NBSA and EBSA respectively. There is no justification, therefore, for assuming that all the 18 imidazole groups, dissociated and undissociated, are available for reaction.

The apparent intrinsic association constants may thus be evaluated from the intercept kn_o and corrected for the charge on the protein molecule.

$$k = k^o e^{-2wZ_p^o} \quad \ldots\ldots\ldots \quad (38)$$

where k is the apparent and k^o the true intrinsic association constant. The values of k^o calculated from the values of k, given in Table VI are assembled in Table XIII. An examination of Table XIII reveals that the intrinsic association constants for the interaction of bovine serum albumins with copper, zinc and cadmium ions agree fairly well with the first association constant of the imidazole with the corresponding metal ion

(see Table V); exceptions seem to be the systems Zn-EBSA and to a lesser extent Cu-EBSA. In the latter systems, however, the binding data and the corrections for the charges on the protein molecule are unreliable. It may, therefore, be concluded that the binding of copper, zinc and cadmium ions, at least in the initial stages of the interaction, takes place at the imidazole groups of the albumin molecule.

Table - XIII

Intrinsic Association Constants
$\log k^{o}$

	Copper 25°C	Zinc 30°C	Cadmium 30°C
EBSA	4.72	3.75	-
NBSA	4.21	3.28	3.27
ABSA	4.52	3.06	3.01

7. The effect of metal uptake on the intrinsic association Constants :

It may be seen from Figs 14 that the binding data cannot be explained in terms of the modified statistical eqn.37. This may be due to a non-equivalence of the reactive sites and/or a participation of competing functional groups. A similar postulate was made for an explanation of the polarographic data reported in Chapter III.

We shall now analyse the equilibrium dialysis data

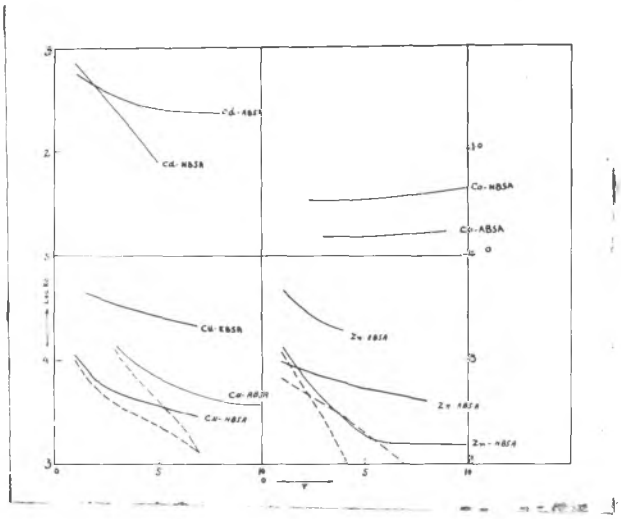

Fig.35 : **Effect of metal uptake on the intrinsic association constants for interaction with the albumins.**
**Full curves refer to imidazole binding alone.*
Dashed curves refer to imidazole binding with corrections for carboxyl binding also.**
(acetate buffer pH 6.5 & μ = 0.20).

*
The intrinsic association constants for the cobalt-serum albumin systems are evaluated on the basis of the 106 carboxyl groups of the protein molecule being the sites responsible for binding the cobaltous ion.

for the binding of copper, zinc and cadmium ions, assuming the imidazole groups to be the seat of reaction. From the eqn

$$r = \frac{n\, k^{o}\, e^{-2wZ_{p}(A)}}{1 + k^{o} e^{-2wZ_{p}(A)}} \qquad (38)$$

it is easy to determine the value of k^{o} as a function of r. Z_p is known from the mobility data (Chapter IV), and n from equation 36. The values of k^{o} thus calculated from the equilibrium dialysis data are plotted in Fig.35 as a function of r, the number of metal ions bound by the protein. It may be noticed that the k^{o} vs r curves of Fig.35 are almost identical with the corresponding curves obtained from polarographic studies (Fig.26).

We may, to start with, examine the system Cu-EBSA and Zn-EBSA. These systems are characterised by practically negligible electrostatic interaction as revealed by the mobility measurements. Furthermore, the competing carboxyl groups are absent. It is, therefore, to be expected that, for these systems at least, the $\frac{r}{A}$ vs r curves should be linear and that the values of k^{o} should be independent of r. This is, however, not the case as may be concluded from Fig.35. Thus one might be tempted to postulate a non-equivalence of the imidazole groups of EBSA. It should, however, be pointed out that the data cover only a narrow binding range and may not be altogether reliable and finally, that the variations in k^{o} are small.

It seems, therefore, that no definite conclusions as to the equivalence or otherwise of the imidazole groups may be

drawn from the system involving the esterified derivative. An analysis of the other systems is rendered difficult because of the competing influence of the carboxyl groups of the protein. These systems are characterised by a fairly large reduction in the intrinsic association constant consequent on the binding of metal ions, the effect being in the order Cd > Zn > Cu for the native protein. For the systems involving ABSA, the effect is nearly of the same order for the three metal ions (Fig.35).

8. Competing influence of carboxyl groups :

If the systems, other than those involving EBSA, are to give any information as to the equivalence of the imidazole groups, it is essential that the competing influence of the carboxyl groups be known. No reliable data on the metal-acetate systems, is however, available. It may be of interest, however, to analyse the equilibrium dialysis data of Klotz and Curme on the binding of copper ions by NBSA at pH 4.0.[15] These authors have shown that the binding of copper ions at this pH occurs essentially at the carboxyl groups of the protein molecule. A value of 2.30×10^3 for k_1 was obtained from their data. The charge on the protein has been assumed to be +20 from mobility measurements at pH 4.0[50]. The number of dissociated carboxyl groups as obtained from eqn.28 comes to about 82. The intrinsic association constant k^o for the binding of the copper ions by BSA at pH 4.0 thus evaluated was 32, which agreed reasonably well with the first association constant of the cupric with the acetate ion (Table V, p. 15).

With a knowledge of the intrinsic association constant for the binding of copper ions by the carboxyl groups of the albumin, we are in a position to evaluate the contribution of the carboxyl groups to the binding of copper ions by bovine serum albumin and its acetylated derivative. The contribution of the carboxyl and imidazole groups of the protein to the binding of metal ions may be represented by the composite equation.

$$r = \frac{(18-n_{HIm})k^{o}_{(MIm)}\cdot e^{-2wZ_p}(A)}{1 + k^{o}_{(MIm)}\cdot e^{-2wZ_p}(A)} + \frac{(106-n_{COOH})k^{o}_{(MCOO)}\cdot e^{-2wZ_p}(A)}{1 + k^{o}_{(MCOO)}\cdot e^{-2wZ_p}(A)} \quad \ldots (39)$$

where the first term represents the contribution of the imidazole groups to the binding process, $(18 - HIm)$ being the number of imidazole groups not covered by a proton at any given moment.

All the 106 carboxyl groups may be assumed to be dissociated at pH 6.5. The second term of eqn.39 may be easily evaluated for a given free metal ion concentration A, with $k^{o}_{(CuCOO)} = 92$, $w = 0.03$ and Z_p as found from the mobility data. Knowing the number (r_{COOCu}) of the metal ions bound by the carboxyl groups and the total number of metal ions bound, we get the number of copper ions (r_{CuIm}) bound by the imidazole groups ($r = r_{CuIm} + r_{COOCu}$). The value of r_{HIm} at a given value of r_{MIm} may be obtained from Fig.33. We can thus calculate k^{o}_{CuIm} at different values of r. The relevant plots are shown in Fig.35 together with those uncorrected for

the contribution of the carboxyl groups towards the binding process.

It may be noticed that, so far as the equivalence of the imidazole groups is concerned, the corrected curves are worse than the uncorrected ones. An examination of the corrected zinc-albumin curves (Fig.35) confering this. In the latter case a $k^{o}_{(ZnCOO)}$ value of 10 was assumed. Similar calculations for the cadmium-albumin systems with k^{o}_{CdCOO} =20 revealed that the binding data at values of r greater than 2 may be best explained in terms of an interaction with the carboxyl groups of the protein alone.

The corrected curves in Fig.35 may be unreliable in that the values of the intrinsic association constants assumed for the calculation of the contribution of the carboxyl groups towards the binding process may be uncertain. It may be seen, however, that even those curves where no such corrections had been made indicated the possibility that the imidazole groups of the protein are only partly available for the binding of metal ions. It is not unreasonable to conclude, therefore, that the participation of the carboxyl groups increases and that of the imidazole groups decreases rapidly as more and more metal ions are bound. This effect increases in the order Cd-NBSA > Zn-NBSA > Cu-NBSA. The relatively low binding of cadmium ions by the esterified derivative is not surprising. The imidazole groups of the albumin molecule do not seem to participate in the binding of cobalt ions as indicated by the

absorption and equilibrium dialysis studies (Chapter II). The intrinsic association constants calculated from the binding data on a basis of the 106 carboxyl groups of the protein being the reactive sites, have an extremely low value, of the order 1.5 - 4.0. Furthermore, the intrinsic association constant is affected but little by the number of metal ions bound (Fig.35).

Gurd and Goodman have investigated the competition between the zinc ion and the hydrogen ion for combining with the imidazole groups of human serum albumin (see p.). These authors have concluded from their studies that the intrinsic association constant for the interaction remained constant at $\log k^{o} = 2.82 \pm 0.05$ over a fairly wide binding range. We have, however, concluded from a parallel analysis of the binding data obtained from the equilibrium dialysis and polarographic measurements that the intrinsic association constant for the interaction of bovine serum albumin with the zinc ion decreases as more and more zinc ions are bound by the protein.

The studies reported by Gurd and Goodman were made in unbuffered solutions containing 0.15M sodium nitrate. In this medium, the zinc ion can be expected to be bound by the serum albumin as the divalent Zn^{++} ion. In the systems studies by us, the uptake of the metal ion must largely involve the MAc^{+} complex ion. Thus, we have a situation in which the imidazole groups of serum albumin are equivalent in their interaction with the Zn^{++} but not with the $ZnAc^{+}$ ion. It may also be concluded that the relative ability of the imidazole

groups for reaction with the MAc^+ ion follows the order CuAc > ZnAc > CdAc > CoAc. It is reasonable to expect, therefore, that the participation of the carboxyl groups in the binding process will, as observed experimentally, follow the reverse order,

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SUMMARY AND CONCLUSIONS

The interaction of copper, zinc, cadmium and cobalt ions with bovine serum albumin and its derivatives has been studied by equilibrium - dialysis, polarographic optical absorption and electrophoretic mobility methods. An acetate buffer (pH 6.5, ionic strength 0.20) was used; giving conditions such that the metal ion could be expected to be present largely as MAc^+ ion.

(1) The affinity of bovine serum albumins has been shown to decrease in the order acetylated protein → native protein → esterified protein, for a given metal ion, and Cu → Zn → Cd → Co for a given protein.

(2) It has been shown that the binding data and the intrinsic association constants calculated from the results of a polarographic study of the metal-serum albumin systems compare in accuracy with those obtained from equilibrium-dialysis.

(3) The metal-protein interactions in buffer solutions have been interpreted in terms of a competition between the metal and hydrogen ions for functional group sites of the serum albumin molecule. The effect of metal uptake on the binding of buffer anions by the serum albumins has also been taken into account.

(4) Electrophoretic mobility measurements have been employed to follow the interaction of metal ions with bovine serum albumins. The expected variations in the net charge on the protein consequent on the binding of metal ions were calculated on the basis of (3) above. A comparison of the

calculated and the experimental mobility data revealed that (a) the cobaltous ion was bound essentially through the carboxyl groups of the protein molecule and (b) copper, zinc and cadmium ions were bound, in the initial stages, through the imidazole groups, the carboxyl groups of the protein molecule contributing materially to the binding of metal ions as more and more metal ions were bound. The effect of the carboxyl groups on the interaction process was found to follow the decreasing order Cd → Zn → Cu.

(5) It has been concluded from a study of the absorption spectra of the metal-protein systems studied that the carboxyl groups of the serum albumin molecule are essentially responsible for binding cobalt and nickel ions.

(6) The results of a quantitative analysis of the equilibrium-dialysis data for the cobalt-serum albumin system were consistent with those obtained from optical absorption and electrophoretic mobility measurements.

(7) A quantitative analysis of the equilibrium-dialysis and polarographic data for the systems involving copper, zinc and cadmium ions has been made in terms of (3) above. The intrinsic association constants evaluated on the basis of the imidazole groups being the reactive sites have been shown to decrease with the number of metal ions bound by the protein. It has been concluded that the availability of the imidazole groups of the serum-albumin molecule for reaction with the MAc^+ ion follows the decreasing order Cu → Zn → Cd.

(8) The competition, optical absorption and mobility

data support the view of the interaction process as outlined in (7) above.

(9) It has been shown that the uptake of copper and zinc ions tended to make the acetylated serum albumin electrophoretically homogeneous.

(10) It has been shown that the constant α , representing the characteristics of the reduction of a metal-protein complex at the dropping mercury electrode, is dependent on the pH of the medium.

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