The Initial Equilibrium Steps in the Interactions of Bovine Plasma Albumin and Zinc(II) Ions. A Potentiostatic Study

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The equilibria between bovine plasma albumin (L) and zinc(II) ions have been investigated at 25° by potentiostatic emf titrations using glass and zinc amalgam electrodes. The ionic medium was 0.5 M $NaClO_4$ and the pH 6.78. The total concentrations of albumin and zinc ions varied through the ranges $0.02-0.5$ mM and $0.1-1.7$ mM, respectively. For each titration the potentiostatic technique involved generating both Zn^{2+} and H^+ ions to constant emf values by using constant current electrolysis. The amount of electricity used was measured by a coulometer.

When the total zinc concentration is plotted against the albumin concentration, the potentiostatic data directly indicate that monomeric albumin complexes predominate at low zinc ion concentrations and that some zinc-albumin polymers exist at high concentrations. The data were further examined using a graphical approach and a computer. The results were found to be consistent with the formation of the monomers ZnL, and Zn_2L, and the series of polymers $ZnL(Zn_5L)_n$ where $n=1$, 3, and 5. The magnitudes of the constants obtained for these polymers indicate that they apparently form *via* zinc ions. It is suggested that this type of polymerization may be of importance in plasma fractionation procedures in which albumin is precipitated by the presence of zinc ions.

The zinc ion appears to be one of the most important metal ions in physiological systems: Zinc ions are present in the active sites of several enzymes, and it is generally held that they are directly involved in the enzymic processes. In blood plasma there are about 0.05 mM zinc ions which are mainly transported as albumin complexes.[1] The affinities of albumin, and some other proteins, for zinc ions have been exploited in plasma fractionation procedures.[2] The proteins can be selectively precipitated at relatively low zinc concentrations. However, little is known concerning the mechanism causing their reduced solubilities.

The main object of this work has been to investigate the initial complexes formed between zinc ions and plasma albumin molecules. On this subject there are obviously disagreements about the number of zinc ions that are bound, although most investigators agree that as far as albumin is concerned, only monomers are involved (Table 1).

Table 1. Survey of investigations on the primary interactions of zinc(II) ions with plasma albumin.

Reference	A mM	B mM	pH and temperature		Medium	Primary binding sites [a]	log K
Tanford [30]	0.18	0.4	5 — 9	(25°)	0.15 M KCl	17	2.9
Saroff and Mark [31]	0.1 — 0.6	0.2 — 0.4	6.1	(26°)	0.1 M NaAc	8	3.1
Kacena [32]	0.01 — 0.4	1.0	8.5	(25°)	0.1 M NaCl	50	3
Rao and Lal [19]	0.15	0.1 — 2.0	6.50	(30°)	0.2 M NaAc	2	3.9
Present work	0.02 — 0.5	0.1 — 1.7	6.78	(25°)	0.5 M NaClO₄	1	9.6 [b]
Gurd and Goodman [8] [c]	0.35	1 — 10	5.5 — 7.5	(0°)	0.15 M NaNO₃	16	2.8
Waldmann-Meyer [33] [c]	—	—	6.07	(25°)	0.15 M NaAc	17	2.8
Perkins [20] [c]	0.1	—	4.5 — 8.5	(—)	0.15 M NaCl	1 — 5	—

[a] All species of type Zn_pL.
[b] Interaction with a thiol group is assumed. An imidazole or a carboxylate group gives 6.5 and 6.4, respectively.
[c] Human albumin, assumed mol.wt. 69 000.

In the present study the data have been treated by methods which are markedly different from those used previously. Thus, an analysis of p and r (the number of Zn^{2+} and albumin molecules (L) bound to the main species) was carried out without introducing any assumptions regarding the compositions of the complexes except that they conform to the general formula,* Zn_pL_r. It is necessary in this analysis that some of the free concentrations can be considered constant, and so the data were recorded potentiostatically. This was carried out by coulometric emf titrations, in which the total concentration of albumin and zinc ions were varied and the free concentrations of Zn^{2+} and H^+ were held constant. Due to the fact that the pH was also held constant throughout the measurements, the treatment of the experimental data did not require considerations concerning the number of protons bound. Thus, the problem of finding the triplets (p, q, r) for the predominating species of general formula, $Zn_pH_qA_r^{(2p+q-ry)+}$, was reduced to one of finding just the pairs (p, r).

Notations

A, total concentration of plasma albumin as defined by eqn. 4; a, molar concentration of free albumin; B, total concentration of Zn^{2+} as defined by eqn. 5; b, molar concentration of free Zn^{2+}; h, molar concentration of free

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* For simplicity, the more general formula for a zinc(II)-albumin complex, $Zn_pH_qA_r^{(2p+q-ry)+}$, is reduced to Zn_pL_r, when the pH is constant. L stands for albumin including all different protonated forms that may enter the complexes (*cf.* eqns. 4 — 6).

H^+; F_{pr}, "formation" constant at constant h as defined by eqn. 6; when a is normalized, a is replaced by aF_{01}^{-1} (eqn. 9) and the constant relating to each species, Zn_pL_r, becomes $F_{pr}F_{01}^{-r}$; p, r, number of Zn^{2+} and albumin molecules (L) bound in Zn_pL_r; $\bar{p}$, $\bar{r}$, average number of p, r;

$$Z_B = (B - b)/A = (\sum_p \sum_q p[Zn_pH_qA])/(\sum_q [H_qA] + \sum_p \sum_q [Zn_pH_qA]) =$$

$$= (\sum_p pb^p F_{p1}F_{01}^{-1})/(1 + \sum_p b^p F_{p1}F_{01}^{-1}) \quad \text{at constant } h \text{ and for } \bar{r} = 1 |$$
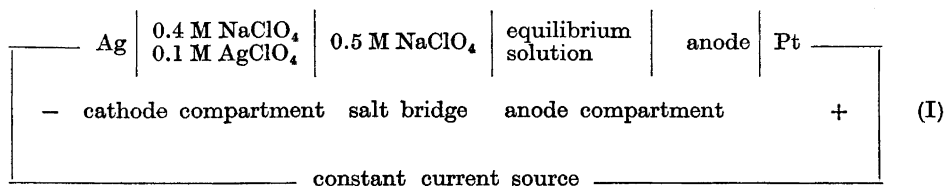
(*cf.* eqns. $3-6$ and eqn. 9).

EXPERIMENTAL

Materials. The crystalline bovine plasma albumin (BPA) used in these studies was an Armour product (Lot No. D 71209). A solution of about 1.5 mM BPA in 0.5 M $NaClO_4$ was freshly prepared for every second titration. A molecular weight of 66 700 was assumed.[3] After filtering the solution (Millipore 0.45 mμ) the pH was increased at 4° by generating OH^- ions at a platinum gauze immersed in the solution.[4] Thereby, the albumin solution is maintained in a reducing environment so that the tendency for half-cystine residues to form disulfide bonds will be minimized. This method avoids a high localized OH^- concentration as well as the impurities of commercial sodium hydroxide.[5] The solution so obtained was dialysed against several portions of 0.5 M $NaClO_4$ of the same pH (2 days at 4°). It was then tested for metal ion impurities and gave a negative result. The albumin in the final solution moved as a single component in sedimentation velocity experiments (Spinco Model E ultracentrifuge at 22° and 52 600 rpm). Further one very faint component was detected by polyacrylamide electrophoresis in 0.1 M $NaClO_4$. The distance moved was what might be expected for albumin dimers. It was estimated that this minor component constituted < 5 % of the albumin. The concentration of albumin was usually determined by measuring the optical density at 279 mμ using $E(1$ %, 1 cm$) = 6.67$ (Ref. 6). This procedure was checked by heating the protein to constant weight at 110° and by a micro-Kjeldahl method.

Zinc perchlorate was prepared by dissolving ZnO (Merck, *p.a.*) in $HClO_4$ and crystallizing the $Zn(ClO_4)_2$ thrice from water. It was also prepared by another route [7] using 99.999 % pure zinc (Fluka AG) and oxygen. These two preparations did not show any measurable differences when the constant, E_{0Zn}, was determined by the procedure to be described later (*cf.* Table 2). The zinc ion concentration of the stock solutions were determined by titrating with EDTA using diphenylthiocarbazone as an indicator. The solutions containing albumin were analysed for zinc ions according to the method described by Gurd and Goodman.[8] The mercury (Grave, *p.a.*) was first subjected to electrolysis in dilute HNO_3 so that small metal impurities could be eliminated. Then it was distilled thrice in the presence of air at 200°. Sodium perchlorate was taken from the batch described in a previous paper.[9] Triply distilled water, freshly prepared in a quartz apparatus, was used throughout.

Method. The equilibria were measured by emf titrations in which A, the total concentration of albumin, and B, the total concentration of zinc ions, were varied. In each titration the free concentrations of both Zn^{2+} (b) and H^+ (h) were held constant. These ions were generated by constant current electrolysis. The equilibrium concentrations were recorded after each generation by using zinc amalgam and glass electrodes.

The electrolysis circuit was

	Ag	0.4 M $NaClO_4$ 0.1 M $AgClO_4$	0.5 M $NaClO_4$	equilibrium solution		anode	Pt	
$-$	cathode compartment		salt bridge	anode compartment			$+$	(I)
			constant current source					

Table 2. The last two parts of a series of experiments. In the first two parts the constant $E_{\Theta G}$,=161.0 mV, was determined and the measuring amalgam (0.016 mol % Zn) was prepared. The generating amalgam (0.015 mol % Zn) has been added before the start of the third part. Data of the solution: volume=24.10 ml; $[Na^+]$=496.9 mM; B=1.573 mM, $[ClO_4^-]$=500 mM.

$\mu_{Zn}{}^a$ $(\mu F/ml)$	$\mu_H{}^a$ $(\mu F/ml)$	BPA 1.455 mM (ml)	E_{Zn} (mV)	E_G (mV)	E_{0Zn} (mV)
−1.422			1463.6	416.1	1461.5
−1.680			1465.7		1461.5
−1.938			1467.7		1461.4
−2.196			1471.2	428	1461.5
−2.456			1475.1		1461.5
−2.716			1480.9		1460.9
−2.972			1493.2		1461.6
−3.018			1495.2	452	1462
		E_{0Zn}=1461.5 mV			
		0.508	1513.7	390.3	
−2.934			1511.2	389.7	
	0.025		1509.6	384.5	
−2.818		1.002	1516.3	389.6	
−2.776			1513.8	388.8	
−2.747			1510.8	387.4	
	0.045		1509.6	384.7	
−2.627		1.512	1516.0	388.9	
−2.587			1511.7		
−2.577			1510.7	386.6	
−2.573	0.056		1509.8	385.3	
	0.062		1509.7	384.9	
−2.463		2.015	1515.0	388.2	
−2.413			1510.7	386.6	
	0.0728		1509.9	385.6	
	0.0788		1509.7	385.1	
	0.0847		1509.6	384.7	
−2.259		2.516	1510.2	386.1	
	0.1008		1509.7	384.8	
−2.120$_5$	0.1162	3.016	1510.7	385.2$_5$	
−2.110$_8$			1510.0	384.9$_5$	
−2.106			1509.7	384.8	
−1.8633	0.1398	4.016	1511.0	386.3	
−1.830			1510.4	385.6	
−1.824	0.1454		1510.1	385.3	
−1.821	0.1510		1509.7	384.7$_5$	
−1.581		5.016	1511.0	386.0	
−1.540	0.1780		1510.6	385.3	
−1.531			1510.1	385.1	
−1.522			1509.7	384.9	
−1.0327	0.2169	7.017 [b]	1510.2	385.3	
−1.0126 [b]	0.2219		1509.8	385.0	
	0.2269		1509.7	384.7	

[a] Generated Zn^{2+} and H^+. [b] Corresponds to A=0.328 mM, B=0.712 mM; analysis gave 0.326 and 0.72 mM.

The anode material was either zinc amalgam or a platinum gauze, the former being situated in a pocket of the titration vessel and the latter near to the surface of the solution. It is assumed that the volume and temperature changes occurring during electrolysis are negligible.[4] The current was passed at a rather slow rate, 0.3 μF/min. In order to avoid a local excess of the ions formed in the vicinity of the anode, the amount of electricity required for each point was added by passing the current in steps. Magnetic stirring on the amalgam surface and bubbling argon through the solution were used as means of obtaining efficient mixing. However, in order to eliminate small amounts of oxygen molecules formed on the platinum gauze when H^+ was generated ($4H^+/O_2$), the magnetic stirring was stopped and the stream of argon increased.

The emf's were measured for cells of the following type

$$ME/\text{equilibrium solution}/0.5 \text{ M NaClO}_4/SE \tag{II}$$
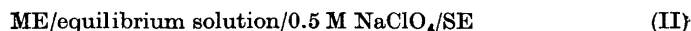
where ME is a zinc amalgam or a glass electrode. SE is the reference Ag,AgCl half cell (containing $NaClO_4$) as described by Biedermann.[10] The equilibrium solution, the reference half cell and the salt bridge were all 0.5 M in ClO_4^-. On this basis it is assumed that the variations of the activity factors are effectively reduced so that all the species in solution may be considered in terms of concentrations.[11] The emf's for the zinc amalgam (E_{Zn}) and glass electrode (E_G) cells may thus be represented by the equations

$$E_{Zn} = E_{0Zn} - 29.577 \log b + E_j \tag{1}$$

$$E_G = E_{0G} - 59.155 \log h + Ej \tag{2}$$

where E_{0Zn} and E_{0G} are constants. The liquid junction potential, E_j, arising at the junction between the equilibrium solution and 0.5 M $NaClO_4$ was approximated by $E_j = +150 \, h$ mV. It is not far from $E_j = +136 \, h$ mV, obtained by the hydrogen half cell.[7]

Emf titrations. Each series of experiments may be divided into four parts: (1) determination of the constant, E_{0G}, (2) preparation of the measuring amalgam, (3) determination of the constant, E_{0Zn}, and ultimate preparation of the generating amalgam, and (4) recording of the data (B, A, b, h).

The constant, E_{0G}, is determined in the absence of both zinc and albumin from a coulometric emf titration with OH^-, using the initial solution of about 5 mM H^+ and the glass electrode cell (cf. Ref. 12). Then in the next step, a zinc(II) solution is added and the measuring zinc amalgam is prepared in a pocket of the titration vessel that has been previously charged with mercury. Both for generating OH^- ions and for preparing the zinc amalgam, the electrolysis circuit (I) is reversed and uses a platinum gauze or mercury (zinc amalgam) as a cathode.

When the zinc(II) concentration in the solution has decreased to a set value, another zinc amalgam (the generating amalgam) is introduced into a second pocket of the titration vessel. In order to keep oxygen contamination to a minimum this amalgam is prepared, immediately before transfer, in a glass vessel connected to the titration vessel by ground glass joints. By using this latter amalgam as the cathode, the zinc(II) concentration is further reduced by stepwise electrolysis until a concentration of 0.05 to 0.1 mM is attained. From the emf's recorded after each step, using the first amalgam as an electrode, the constant for the amalgam cell, E_{0Zn}, is calculated (Table 2). The final concentration of zinc in this second zinc amalgam usually became 0.02 to 0.04 mol %.

Finally, an albumin solution having $-\log h > -\log h_0$, is successively added and h and b are held constant at h_0 and b_0. This is arranged by generating Zn^{2+} and H^+ in steps (circuit I) so that h and b become equal to h_0 and b_0. The values of h and b are checked after each step by measuring the emf's of the zinc amalgam and glass electrode cells (II). An example of the procedure is shown in Table 2. It should be noted that there was a decrease in the pH when the zinc ions were generated into the albumin solutions containing Zn^{2+}.

We also attempted to investigate the equilibria by varying B as a function of b for constant h and constant A. The first three parts of this series of experiments were carried out as described above. Then samples of an albumin solution were added to the reaction vessel until the concentrations A_0 and h_0 were obtained. Then finally the zinc ion concentration was increased successively by using a titrant containing 0.05 M $Zn(ClO_4)_2$ and 0.4 M $NaClO_4$. After each addition of Zn^{2+}, the value of h was adjusted to the constant value, h_0, using two albumin solutions having $A = A_0$ and h-values partly above and

partly below h_0. The ultimate adjustment of h was usually made by generating H^+ by the circuit (I). The values of h and b were obtained from eqns. 1 and 2 using emf's recorded by cells of the type II.

The A range was $0.02-0.5$ mM and B was $0.1-1.7$ mM. In an attempt to extend the B range by using the titration procedure of the second type described we obtained a precipitate of albumin at $B\sim8$ mM and $A = 0.35$ mM. The precipitate could be dissolved by diluting the solution with 0.5 M $NaClO_4$. The emf's usually attained constant values within 20 min when the concentration of free Zn^{2+} was higher than 10 μM; however, at low values of B the readings were constant only after 1 to 2 h. The potentials then remained constant within $0.1-0.2$ mV for more than 12 h. At the end of each titration, the solution was frozen and kept at $-20°$ until it was analysed for zinc ions and albumin.

Apparatus. All the experiments were made in a closed apparatus, and the titration vessel was connected by ground glass joints to inlets for electrodes and argon gas (inlet and outlet). This apparatus was immersed in a paraffin oil thermostat kept at $25.00 \pm 0.01°$ in a room thermostated at $25.0 \pm 0.1°$. Oxygen was carefully excluded by bubbling argon through the solution. The argon was freed from O_2 by hot activated copper and then passed through solutions of 10 % NaOH, 10 % H_2SO_4, 0.5 M $NaClO_4$, and finally 0.5 M $NaClO_4$ within the oil bath. Attempts were made to reduce the oxygen content of the titrants by pretreating them with this purified, bubbling argon.

The glass electrodes, Beckman No. 40498, were calibrated against a hydrogen electrode. The emf's were measured by digital voltmeters (Digital Measurements, type DM 2022S). They were calibrated by a Leeds and Northrup compensator type K2 using a Weston standard cell. A similar cell was also connected across the zinc amalgam electrode in order to reduce the magnitude of the emf's measured. The constant current source (*cf.* I) was manufactured by Electronic Measurements (No. C 612). It was calibrated before every second or every third measurement. The variations in the currents between two consecutive calibrations were usually less than 0.3 ‰. All pipettes and burettes were calibrated using water and 3.0 M $NaClO_4$.

Table 3. Experimental data $(B,A)_{b,h}$ and $B(b,A)_h$ for $-\log h = 6.782$. The points used in the Letagrop refinement are indicated by $\Delta B = (B_{calc} - B_{exp})$ and their sign. In calculating B_{calc} we used Set 3 of Table 4 and the δB values listed in the head of each titration.

Table 4. Results of Letagrop treatment, data $B(A)_{b,h}$.

No.	$10^{-6} \times$ $F_{11}F_{01}^{-1}$	$10^{-11} \times$ $F_{21}F_{01}^{-1}$	$10^{-19} \times$ $F_{41}F_{01}^{-1}$	$10^{-35} \times$ $F_{62}F_{01}^{-2}$	$10^{-90} \times$ $F_{16,4}F_{01}^{-4}$	$10^{-144} \times$ $F_{26,6}F_{01}^{-6}$	$10^2\sigma(B)$	$10^9 U$
1	2.4 ± 0.2	1.5 ± 0.2	—	2.0 ± 0.2	1.8 ± 0.4	—	2.1	30
2	2.44 ± 0.10	1.2 ± 0.1	—	1.96 ± 0.11	0.66 ± 0.15	1.0 ± 0.2	1.6	19
3 [a]	2.31 ± 0.08	1.35 ± 0.1	—	1.70 ± 0.08	0.64 ± 0.09	1.2 ± 0.2	1.0	7.6
4	2.0 ± 0.2	3.9 ± 0.4	7.6 ± 0.8	—	—	—	3.0	64
5 [a]	1.8 ± 0.2	3.5 ± 0.4	8.3 ± 0.7	—	—	—	1.9	24

[a] δB varied.

TREATMENT OF DATA

The two sets of primary data, $(B,A)_{b,h}$ and $(B,b)_{A,h}$, are shown in Table 3 and are represented graphically in Fig. 1. The data $(B,A)_{b,h}$ were first analysed by graphical methods and then by using a computer and the general minimizing computer program, Letagropvrid.[13] It was assumed that the equilibria in the zinc(II)-albumin system can be described by the general reaction

$$p\mathrm{Zn}^{2+} + q\mathrm{H}^+ + r\mathrm{A}^{y-} \rightleftharpoons \mathrm{Zn}_p\mathrm{H}_q\mathrm{A}_r^{(2p+q-ry)+} \qquad (3)$$

and the equilibrium constant, β_{pqr}.

The results obtained were found to be consistent by the formation of the monomers ZnL and Zn_2L, and the polymers $\mathrm{Zn}_6\mathrm{L}_2$, $\mathrm{Zn}_{16}\mathrm{L}_4$, and $\mathrm{Zn}_{26}\mathrm{L}_6$. The corresponding constants, $(F_{pr}F_{01}^{-r})$, are shown in Table 4 (Set 3), and their logarithms on p. 3837.

The potentiostatic method

For a constant value for h we obtain from mass balances:

$$A = a + \sum_q\sum_r rh^q a^r \beta_{0qr} + \sum_p\sum_q\sum_r rb^p h^q a^r \beta_{pqr} = \sum_r ra^r F_{0r} + \sum_p\sum_r rb^p a^r F_{pr} \qquad (4)$$

$$B = b + \sum_p\sum_q\sum_r pb^p h^q a^r \beta_{pqr} = b + \sum_p\sum_r pb^p a^r F_{pr} \qquad (5)$$

where

$$F_{pr} = \sum_q h^q \beta_{pqr} \qquad (6)$$

From measurements at different levels of h one may, for each level, deduce the most important values of p and r using eqns. 4 and 5. Then, in principle, the variation of the F_{pr} with h (eqn. 6) enables us to deduce the prevailing values of q.

For constant values of both h and b, eqns. 4 and 5 reduce to the relationships

$$A = \sum_r ra^r G_r \qquad (4a)$$

$$B = b + \sum_r a^r S_r \tag{5a}$$

where

$$G_r = \sum_{p \geq 0} b^p F_{pr}; \quad S_r = \sum_{p \geq 1} p b^p F_{pr} \tag{7,8}$$

Now, if r is equal to unity, eqns. 4a and 5a may be written

$$A = a G_1; \quad B = b + a S_1 = b + A \cdot \text{constant} \tag{4b,5b}$$

It follows from eqn. 5b that a straight line is obtained if B is plotted against A with h and b constant.

<center>G r a p h i c a l   a n a l y s i s</center>

It is shown by Fig. 1 that the data follow straight lines for high $-\log b$ but concave curves for low $-\log b$. This indicates that mononuclear albumin complexes, $r = 1$, predominate at high $-\log b$ (eqn. 5b). From the plot, Z_B against $\log b$, these mononuclear species were found to be ZnL and Zn_2L. By comparison to normalized graphs [14] we obtained the following values for the logarithm of the constants $F_{11} F_{01}^{-1}$ and $F_{21} F_{01}^{-1}$:

$$\log (F_{11} F_{01}^{-1}) = 6.3; \quad \log (F_{21} F_{01}^{-1}) = 11.15$$

In order to analyse the equilibria at low $-\log b$, where polynuclear albumin complexes may exist, we first calculate $\log \mathbf{a}/A$ as defined by

$$\log \mathbf{a}/A = \log a F_{01}/A = \log \left((a \sum_{q \geq 0} h^q \beta_{0q1})/A \right) = \log a/a_0 \tag{9}$$

The quantity $\log a/a_0$ is given by the equation

$$\log a/a_0 = - \int_{-\infty}^{\log b} (\delta B/\delta A)_{b,h} \, \mathrm{d} \log b \quad \text{at } A, h \text{ constant} \tag{10}$$

This relationship is derived in the same manner as has been previously described for similar equations [12,15] (cf. Ref. 16). It is assumed in eqn. 9 that all the acid-base species $H_q A_r$ can be approximated to be of the mononuclear $H_q A$ type.

In the next step we calculate the values of p and r for the predominating species $Zn_p L_r$, using the equations

$$p = (\varDelta \log (B-b)/\varDelta \log b)_{h,\mathbf{a}} \tag{11}$$

$$r = (\varDelta \log (B-b)/\varDelta \log \mathbf{a})_{h,b} \tag{12}$$

For high B, most of the albumin exists as zinc complexes. The value of p may then be calculated from the equation

$$p = r(\varDelta B/\varDelta A)_{b,h} \tag{13}$$

Eqns. 11 − 13 presuppose that in each concentration range considered there is only one main complex in the solution.

Fig. 2 shows that the data, $\log (B-b)$ against $\log \mathbf{a}$, can be fitted to straight lines. Their slopes, r, increase with b and attain values from 1 to 6. When the values of p are plotted against these r's in a $(\bar{p}; \bar{r})$ diagram, a great deal of the

data follow the line, $p = 5r - 4$ (Fig. 3). The complexes formed in the solution must then have the composition, $Zn_{-4}(Zn_5L)_n$ with values of n from 1 to 6.
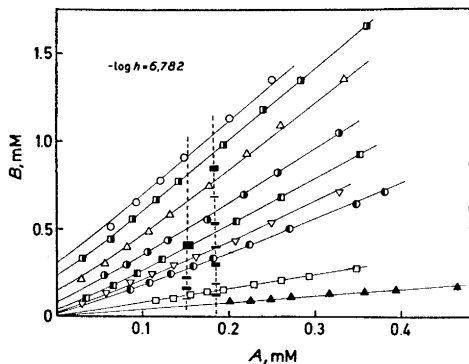

Fig. 1. Zinc(II) complexes of plasma albumin in 0.5 M $NaClO_4$ at 25° and $-\log h = 6.78_2$. The symbols are the experimental points and the curves have been calculated assuming the equilibrium constants of Table 4 (Set 3). For data $B(A)_{b,h}$ the values of $-\log b$ are: O, 3.515; ■, 3.63; △, 3.83; ◑, 4.10; ◪, 4.405; ▽, 4.63; ◐, 4.78; □, 5.93; ▲, 6.585. For data $B(b,A)_h$ symbols, ▬, of heights equal to $B_{\text{calc}} - B_{\text{exp}}$.
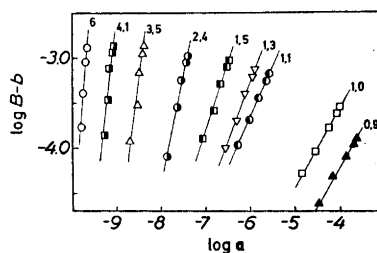
Fig. 2. The determination of r, the prevailing number of albumin molecules bound in the complex Zn_pL_r. The data, $\log (B-b)$ against $\log$ **a**, have been fitted to straight lines, and the slopes are indicated (cf. eqn. 12). For symbols, see legend to Fig. 1.
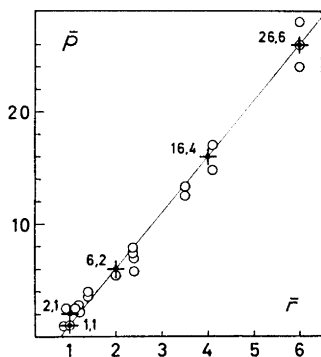
Fig. 3. The average numbers of zinc ions ($\bar{p}$) and albumin molecules ($\bar{r}$) bound in complexes Zn_pL_r. Points corresponding to predominating species are indicated by their pairs of (p,r). Note that all points except (2,1) fall on the straight line, $p = 5r - 4$.

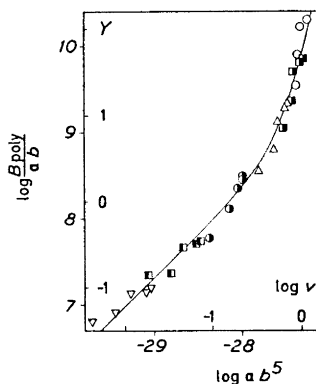Fig. 4. Data $\log B_{\text{poly}}/\textbf{a}b$ against $\log \textbf{a}b^5$ of eqn. 14 compared with the model function, $Y = \log (6v + 16v^3 + 26v^5)$. For symbols, see legend to Fig. 1.

When the data, $\log B_{poly}/ab$ against $\log ab^5$, were compared to curves calculated from normalized model functions, $Y(\log v)$, it was found that these data could be explained by only two parameters (Fig. 4). The equation

$$\log B_{poly} - \log ab = \log (B - b - abF_{11}F_{01}^{-1} - 2ab^2F_{21}F_{01}^{-1}) - \log ab$$
$$= \log (6ab^5K_0 + 16(ab^5)^3K_0K + 26(ab^5)^5K_0K^2) \tag{14}$$

was normalized to

$$Y = \log (6v + 16v^3 + 26v^5) \tag{15}$$

In eqn. 14 a series of complexes, $Zn_{6+10s}L_{2+2s}$ with $s = 0$, 1, and 2, is assumed. The constant, K, for the addition of a new dimer unit, $Zn_{10}L_2$, is assumed to be independent of s for $s \geq 0$. Hence, the constants corresponding to Zn_6L_2, $Zn_{16}L_4$, and $Zn_{26}L_6$ of the series may be written K_0, K_0K, and K_0K^2. This also defines the constant K_0. The variables $(Y, \log v)$ of eqn. 15 are logarithms of products containing $(B_{poly}/ab, ab^5)$ and the constants $(K^{1/2}K_0^{-1}, K^{1/2})$. In the position of the best fit these constants are obtained from the differences in readings on the calculated and experimental scales (Fig. 4). For $s = 0$, 1, and 2 this gave

$$\log F_{6+10s,2+2s}F_{01}^{-(2+2s)} = \log K_0 + s \log K = 35.5 + s \ 54.6$$

REFINEMENT OF CONSTANTS

The data were treated with the second version of the program Letagrop.[13] We searched for these values of the constants, F_{pr}/F_{01}^r, that minimize U, the error square sum:

$$U = \sum (B_{calc} - B_{exp})^2 \tag{16}$$

The special block, "Ubbe", of this program was rewritten for the present problem. From the data ($\log b$, A) the computer first calculated the quantity, a, using the preliminary constants $F_{pr}F_{01}^{-r}$. The first constants were those obtained graphically. The machine then computed the quantity $a^rb^pF_{pr}F_{01}^{-r}$ corresponding to each species Zn_pL_r and finally B_{calc}. During this treatment we attempted to explain the data by sets of other species having compositions rather close to the graphical ones. We also assumed that in each series of experiments there is a constant error, δB, which may be due to, for instance, analytical errors in B and A or to errors in the constants, E_{0G} and E_{0Zn} (Ref. 13).

It is shown by Table 4 that we have to assume both the polymers Zn_6L_2, $Zn_{16}L_4$, and $Zn_{26}L_6$ and the monomers ZnL and Zn_2L in order to explain the data (Set 2). When these polymers are replaced by the monomer Zn_4L there are marked increases both in $\sigma(B)$ and in the error square sum, U, whether the systematic errors are considered or not (Sets 4 and 5). For the set including polymers (Set 3) these systematic errors were about 1 % compared to the 10 % for Set 5. Also, when we attempted to introduce the monomers Zn_3L and Zn_5L into Set 4 we neither obtained any significant reduction in $\sigma(B)$ nor in U. Their constants came out with large errors. This was also the case when the species Zn_3L, Zn_4L, and Zn_5L were introduced into Set 2.

When a number of dimeric species ($r = 2$) having values of p equal to 1, 2, 5, and 7 were successively tested in Set 2 and the constants varied together, there was not an improved agreement with the experimental data. Some

of these dimeric species were rejected by Operation Miko of the program.[13] Others came out with constants having very large errors. Thus, the present data do not provide evidence concerning the possible presence of dimers in the albumin stock solutions, even though commercial albumin preparations are known to contain a certain amount of dimers.[17] If these do exist, we may conclude that their concentrations are not greater than 5 % (cf. p. 3829). It should also be born in mind that our albumin solutions were not subjected to pH's lower than 5.5 (cf. Ref. 18), i.e. they were prepared under experimental conditions that might reduce side reactions of sulfhydryl groups.

When recalculated to log $(F_{p,r}F_{01}^{-r})$ and giving 3 σ limits, Set 3 becomes

$$\log F_{11}F_{01}^{-1} = 6.36 \pm 0.05, \qquad \log F_{21}F_{01}^{-1} = 11.1 \pm 0.1,$$

$$\log F_{62}F_{01}^{-2} = 35.23 \pm 0.07, \qquad \log F_{16,4}F_{01}^{-4} = 89.8 \pm 0.2,$$

$$\log F_{26,6}F_{01}^{-6} = 144.1 \ (< 144.3)$$

As an important test of the accuracy of our calculations let us consider Fig. 1 and Table 3. They show that the data of the present accuracy may be satisfactorily described by Set 3 of Table 4.

Fig. 5 shows the distribution of zinc ions between the different species as a function of $-\log b$ at pH 6.78. In this system the distribution is also a function of the albumin concentration. The values of A in Fig. 5 are the border values for the range studied.

Fig. 5. Distribution of zinc(II) ions between different complexes at pH 6.78 expressed as moles per 100 moles. The assumed constants were Set 3 in Table 4. For a certain $-\log b$, the moles per 100 moles of zinc ions present as a given species are represented by the segment of the vertical line falling within the corresponding range. The minor species are indicated by their p,r values in the formula Zn_pL_r. *Full drawn curves,* experimental range; *broken curves,* extrapolated range.
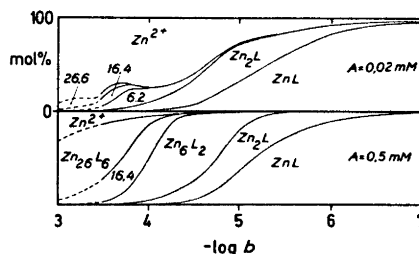
DISCUSSION

The calculations presented in the previous section have furnished evidence for the formation of the monomeric species ZnL and Zn_2L and for the presence of the polymers Zn_6L_2, $Zn_{16}L_4$, and $Zn_{26}L_6$. The existence of Zn_2L has been reported previously by Rao and Lal.[19] Our data agree with the suggestion of these authors that the albumin molecule has two binding sites for zinc ions which form much stronger complexes than other binding sites. However, our data also indicate that there is a definite difference in stability between the first and the second association steps. Thus, at low concentrations of both albumin and zinc ions the species ZnL predominates (Fig. 5). It appears to be the most important species in the concentration ranges of human blood plasma, $A = 0.6$ mM, $B = 0.05$ mM (Figs. 1 and 5). (Human and bovine albumins have rather similar compositions and properties (cf. Refs. 18, 20).)

When the zinc ion concentration becomes about 0.5 mM in the present concentration range of albumin, the albumin molecules tend to condense into polymers of composition $ZnL(Zn_5L)_n$ (Figs. 1 and 5). Of these, we have identified Zn_6L_2, $Zn_{16}L_4$, and $Zn_{26}L_6$, and we may assume that the process continues further for higher zinc ion concentrations. This is substantiated by the fact that albumin causes precipitates in 0.5 M $NaClO_4$ medium at zinc ion concentrations as low as 8 mM (p. 3832). Thus, polymerization might be of importance in fractionation procedures, where zinc ions are used as precipitants for albumin.[2]

There is a decrease in pH when zinc ions react with albumin (Table 2). This indicates that the complex formations involve groups that are, if only partly, protonated at pH 6.8. Among these groups present in albumin we might tentatively assume that the zinc ions are bound to the single thiol and α-amino groupings and also to some of the imidazoles. The thiol group of albumin is known to form very stable complexes with mercury(II) ions (for a review, see Ref. 18). Cupric ions, on the other hand, form their most stable complex with a site involving the amino terminal aspartyl residue.[21] The imidazole groups of albumin have repeatedly been assumed to be important ligands for zinc ions.[8,22] Also, it has recently been shown that in the crystalline state, sperm whale myoglobin binds a single zinc ion at an imidazole group.[23]

As a first approximation we may assume that only one proton is set free for each zinc ion bound. The magnitudes of the zinc association constants for the monomers may then be estimated from the relationships

$$K_{11} = F_{11}F_{01}^{-1}hK_i^{-1} \; ; \; K_{21} = F_{21}F_{11}^{-1}hK_i^{-1}$$ (17 a, b)

where K_i is the intrinsic dissociation constant for the acid-base group involved. The F-terms are assumed to differ only in regard to the association of a zinc ion. The values of the K_i-constants can be estimated to be $K_i(SH), = 10^{-10.0}$ Ref. 24), and $K_i(\alpha\text{-}NH_3), = 10^{-7.9}$, $K_i(Im), = 10^{-6.9}$ (Refs. 25, 26). Thus, if imidazole groups are assumed, both K_{11} and K_{21} become about three powers of ten greater than the constant reported for the 1 : 1 zinc complex of imidazole ($10^{2.6}$; Ref. 27). The thiol group, on the other hand, gives a K_{11}-value, $10^{9.6}$, which is not very far from the K_1-constants reported for Zn^{2+}-cystein ($10^{9.9}$; (Ref. 28) and Zn^{2+}-mercaptoacetic acid ($10^{8.0}$; Ref. 29). Likewise, the introduction of $K_i(\alpha\text{-}NH_3)$ into eqn. 17b gives a K_{21}-value of $10^{5.8}$. This is the same order of magnitude as the values of K_1 reported for zinc(II) chelates of amino acids ($10^{5.5}$; Ref. 24). Hence, the second zinc association step might involve a bidentate complex, for example formed via the α-amino and β-carboxylate groups of the albumin aspartyl residue (cf. Ref. 19).

If we then extend this procedure to the formation of polymers, we arrive at a conclusion that they form through zinc ions. For example, if the zinc ion binding was independent of the formation of Zn_6L_2, the association constant for the last two zinc ions becomes

$$K_{ZnIm} = (F_{62}F_{21}^{-2}h^2K_{i(Im)}^{-2})^{1/2} = 10^{6.6}$$

This is almost four orders of magnitude greater than the constant reported for zinc-imidazole,[27] and hence quite improbable. As currently used in the analysis of titration data for proteins we have assumed that the interactions

of the actual imidazole groups with H^+ or Zn^{2+} are identical and independent of each other. One obvious approach to reduce the value of the constant K_{ZnIm} is to assume that the single thiol groups of both molecules are equally strongly bound to the same zinc ion. Then, we obtain $10^{4.3}$ for K_{ZnIm}. A further reduction is obtained if a second zinc ion bridge is assumed to be formed by coordination through an imidazole group from each molecule. Provided that K_{ZnIm} is approximately equal to $K_{Zn(Im)_2}$ the value then becomes $10^{3.4}$.

Similarly, if the formation of $Zn_{16}L_4$ from Zn^{2+} and two Zn_6L_2 would be independent of the zinc ions then

$$K_{ZnIm}' = (F_{16,4}F_{6,2}^{-2}h^4K_{i(Im)}^{-4})^{1/4} = 10^{5.0}$$

whereas the mediation of this association process by two zinc ions gives $K_{ZnIm}' = 10^{3.4}$. Each zinc ion is assumed to be bound through two imidazoles coming from different dimers. Using the same assumptions, we may also describe the formation of the zinc-imidazole complexes in $Zn_{26}L_6$ with almost the same constant, $K_{ZnIm}' = 10^{3.3}$, provided that a dimer and a tetramer are bound together *via* two zinc ions. In this hexamer, four of the sixteen available imidazole groups will bind zinc ions. The six albumin molecules will be linked together by ten zinc ions.

In conclusion, it may be remarked that the values of our F constants (Set 3 in Table 4) indicate that the zinc ions induce the formation of albumin polymers. This is supported by the fact that albumin specifically precipitates at a relatively low zinc ion concentration (*cf.* p. 3832 and Ref. 2). It is also widely known that mercury ions form albumin dimers by complex formation *via* the single thiol group of each molecule.[18] However, it is only when two zinc ions are assumed to be involved in each of the association steps, $ZnL \rightarrow Zn_6L_2 \rightarrow Zn_{16}L_4 \rightarrow Zn_{26}L_6$, that we obtain formation constants for zinc-imidazole that are of the same orders of magnitude as the K_1 constant for the corresponding low-molecular-weight complex.[27]

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