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A Calorimetric Investigation of the Copper–Bovine Plasma Albumin Interaction†

Fred H. Reynolds, Jr.,‡ R. K. Burkhard,* and Delbert D. Mueller

ABSTRACT: The thermodynamics of the copper–bovine plasma albumin interaction was investigated by a combination of microcalorimetry and equilibrium dialysis. Computer regression procedures were used to calculate the thermodynamic parameters for the first 10 steps of the reaction. At 20° in sodium acetate buffer, $I = 0.1$, pH 4.80, the enthalpy changes ranged from 4.0 to 2.2 kcal mol^{-1} for the first to the tenth bound ion, respectively. Similarly, the apparent standard free-energy changes ranged from -5.0 to -2.7 kcal mol^{-1} and the entropy changes from 31 to 17 cal mol^{-1} deg^{-1}. Two to three protons were released per mol of copper bound during the binding of the first 4 mol of copper. Hydrogen–deuterium exchange studies suggested that no conformational change occurred when 1 mol of copper was bound but did occur when 10 mol of copper was bound.

The thermodynamic parameters of the copper–bovine plasma albumin interaction were determined more than two decades ago from equilibrium dialysis data taken at two different temperatures (Klotz and Curme, 1948). The development of sensitive microcalorimeters, however, now permits direct measurement of the enthalpy changes associated with this interaction. Accordingly, a combination of calorimetric and equilibrium dialysis techniques should provide the desired thermodynamic parameters from data taken at one temperature. This paper reports the results of such a study and compares them to those reported previously. To aid interpretation of the data, studies were also performed regarding the effect of acetate ion, proton evolution, and conformational changes. The results from these are also reported.

Materials and Methods

Bovine Plasma Albumin Samples. Crystalline bovine plasma albumin (Pentex Corp., now Research Division, Miles Laboratories, Inc., lot nos. 15, 16 and 18) was used without further purification for most of the studies. Some albumin was purified, however, by defatting (Sogami and Foster, 1968) and deionizing (Dintzis, 1952).

Reaction Conditions. All reactions were performed at 20°. Generally, solutions were prepared in a sodium acetate buffer, $I = 0.1$, pH 4.80. In one set of experiments the molarity of the acetate ion in the buffer was varied to determine what effect this might have on the copper–albumin interaction.

Equilibrium Dialysis. Visking dialysis tubing (Union Carbide) was washed in boiling deionized water and soaked in buffer overnight before use. Dialysis bags (2 ml) were suspended in 8-ml volumes of buffer solution containing reagent grade cupric acetate and shaken for 18 hr. The albumin concentration used for these studies was 2.5%. Copper was assayed spectrophotometrically as the 2,9-dimethyl-1,10-phenanthroline chelate (Smith and McCurdy, 1952).

Microcalorimetry. A heatburst microcalorimeter similar to that described by Kitzinger and Benzinger (1960) was used to measure heat changes. The heat associated with the interaction of copper with albumin was obtained by mixing, in one of the bicompartmented vessels, a solution of 5% albumin with one of cupric acetate, while in the reference vessel a similar solution of albumin was mixed with acetate buffer. The heat of dilution of the cupric acetate was measured separately by mixing cupric acetate solutions with equal volumes of acetate buffer in the reaction vessel, while two equal volumes of acetate buffer were mixed in the reference vessel.

Analysis of Binding Data. Binding data were analyzed by use of eq 1 (Scatchard, 1949) which is applicable to processes involving sites that can be grouped into classes having similar intrinsic binding constants

$$\bar{\nu} = \sum_{j=1}^{m} \frac{N_j k_j[\mathrm{A}]}{1 + k_j[\mathrm{A}]} \qquad (1)$$

where $\bar{\nu}$ is the average number of mol of ligand bound per mol of protein, [A] is the equilibrium molarity of the unbound ligand, m is the number of classes of sites, N_j is the number of sites in class j, and k_j is the intrinsic constant for class j. This analysis was done by use of a computer program developed at the Division of Computer Research and Technology of the National Institutes of Health (Fletcher and Spector, 1968; Fletcher *et al.*, 1970). Subsequently, N_j and k_j values were used to find K_{ij} for each step of the reaction from eq 2 (Klotz, 1946), where K_{ij} is the association constant

$$K_{ij} = \frac{N_j - (i-1)}{i} k_j \qquad (2)$$

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‡ Present address: Institute for Cancer Research, College of Physicians and Surgeons, Columbia University, New York, N. Y. 10032.

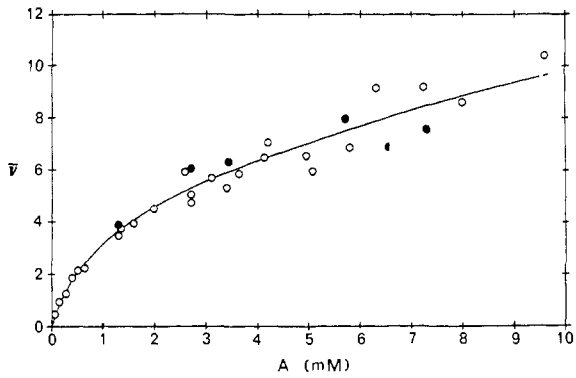

FIGURE 1: The binding of copper to bovine plasma albumin, expressed as $\bar{\nu}$ *vs.* the concentration of unbound copper (A) in equilibrium with that which is bound. A includes all forms of unbound copper (Cu^{2+}, $CuOAc^{+}$, and $Cu(OAc)_2$). The filled circles represent data from samples of crystalline albumin that had been subjected to further purification. The line is calculated from eq 1 using $m = 2$, $N_1 = 4$, $N_2 = 20$, $k_1 = 1372$, and $k_2 = 43.53$. Albumin concentration 2.5%, pH 4.80 (acetate buffer, $I = 0.1$), 20°.

for step i in class j. Each K_{ij} thus obtained, after arranging in decreasing order, was redesignated as a K_i.

Results from computer fits reported in this paper are those that had the smallest root-mean-square errors (RMS errors).[1] If two fits had similar RMS errors, then the one reported was the one with the smaller number of adjustable parameters. The definition of RMS error used for the above computer fits was

$$\text{RMS error} = \left[\frac{\sum_{i=1}^{n}(y_i - y)^2}{\sum_{i=1}^{n} w_i}\right]^{1/2} \quad (3)$$

where y_i represents the measured quantity involved, y the corresponding calculated value, w_i and n, the statistical weight and number of measured quantities, respectively. Each data point was weighted as a normalized reciprocal of its standard deviation. All computer calculations used an IBM 360/50 computer.

Analysis of Calorimetric Data. To calculate ΔH_i corresponding to each K_i it was necessary to relate $\bar{\nu}$ to the total copper concentration. This was accomplished by the above-mentioned computer program. In addition, this program was used to relate Q, the quantity of heat taken up by the reaction, to $\bar{\nu}$ according to eq 4 where C_1 and C_2 are constants. The

$$Q = \frac{C_1 C_2 \bar{\nu}}{1 + C_2 \bar{\nu}} \quad (4)$$

first derivative of this equation then gives the enthalpy change for any selected $\bar{\nu}$, as would any other equation relating the two quantities.

Proton Titration. The protons evolved when copper reacts with albumin in unbuffered systems were titrated with a microburet and the neutralization followed with a Beckman Century SS expanded-scale pH meter using a single electrode (Beckman No. 39030). Each reaction was initiated by mixing 10 ml of albumin solution with 10 ml of cupric acetate solution both initially at pH 4.80. All solutions were made from boiled deionized water, analyzed under nitrogen, and corrected for dilution.

Hydrogen–deuterium exchange was followed in the near infrared region using a Cary 14R recording spectrophotometer (Scarpa *et al.*, 1967; McBride-Warren and Mueller, 1972). Protein exchange rates in this region can be obtained by following either HOD production at 1.41 μ or N–H decrease at 1.50 μ with time, as shown by the following overall reaction: Pro-N-H + $D_2O \rightleftharpoons$ Pro-N-D + HOD. In an overwhelming excess of D_2O the reaction becomes pseudo-first order in the forward direction. Exchange kinetics in proteins, however, follows a sum of first-order rates due to the presence of several classes of labile peptide hydrogens (Hvidt and Nielsen, 1966).

Exchange was conducted in deuterium oxide (99.8 mol % D; Bio-Rad Laboratories) solutions containing acetate buffer at pD 4.76 and sufficient cupric acetate to give the desired $\bar{\nu}$'s. After equilibration at 20.5 ± 0.5° in the spectrophotometer, the D_2O in the 5-cm sample cell was used to make a 2.5% solution of crystalline albumin. Upon returning the solution to the spectrophotometer cell, spectra were recorded at selected intervals over a 6-hr reaction period. Absorbances at 1.41 and 1.50 μ were scaled to readings at 1.18 μ, a region virtually independent of exchange, to correct for minor fluctuations in the early spectra. Absorbance readings at complete exchange were obtained by adding either NaOD in D_2O to give pD ~10 or DCl in D_2O to give pD ~2. The same amount of NaOD or DCl was added to the reference cell to compensate for any HOD in these solutions. In either case the rates are greatly accelerated and no further exchange was noted after 24–48 hr.

Results

Equilibrium Constants and Free-Energy Changes. $\bar{\nu}$ was calculated for each value of A using 66,700 as the molecular weight of albumin (Squire *et al.*, 1968). When $\bar{\nu}$ was plotted *vs.* A (Scatchard, 1949) the relationship was seen to be nonlinear and hence two classes of binding sites were used in the initial computer fits. As a result of many attempts to fit the data, it was found that a model having $m = 2$, $N_1 = 4$, $N_2 = 20$, $k_1 = 1372$, $k_2 = 43.53$, and a RMS error of 0.34 could not be improved by adding more sites and/or more classes of sites. The experimental data and the curve calculated for this model are shown in Figure 1. The equilibrium constants and corresponding free-energy changes for the first ten steps of the reaction according to this model are listed in Table I. To avoid possible confusion it should be mentioned that these free-energy changes are apparent standard free-energy changes for the reaction between copper and albumin in an acetate buffer of pH 4.80 and $I = 0.1$. Each has been designated as a $\Delta G^{\circ}{}_i'$ (pH 4.80) were i denotes the particular step in the overall reaction. Similar notations have been used for the other thermodynamic data.

Enthalpy and Entropy Changes. The plot of Q *vs.* $\bar{\nu}$ and the best computer fit of these data to eq 4 are shown in Figure 2. The constants C_1 and C_2 in this equation were 105 and 0.0416, respectively. Using these in the mathematical expression for $dQ/d\bar{\nu}$ the enthalpy changes for the first ten steps of the copper–albumin interaction were calculated. These and the corresponding entropy changes are shown in Table I.

[1] The mathematical definition of RMS error for binding and calorimetric calculations differs from that for H–D exchange calculations. The definition of each can be found in the text. pD represents the negative logarithm of deuterium ion (D^+) concentration and is computed as pD = pH meter reading + 0.4.

TABLE I: Apparent Equilibrium Constants and Thermodynamic Parameters for the First Ten Steps of Copper–Bovine Plasma Albumin Interaction (pH 4.80, I = 0.1, 20°).[a]

i	K_i	$\Delta G°_i'$ (pH 4.80), kcal mol^{-1}	$\Delta H°_i'$ (pH 4.80), kcal mol^{-1}	$\Delta S°_i'$ (pH 4.80), kcal mol^{-1} deg^{-1}
1	5488	−5.0	4.0	31
2	2058	−4.4	3.7	28
3	914.7	−4.0	3.4	25
4	870.4	−3.9	3.2	24
5	413.4	−3.5	3.0	22
6	343.0	−3.4	2.8	21
7	261.1	−3.2	2.6	20
8	185.0	−3.0	2.4	19
9	139.3	−2.9	2.3	18
10	108.8	−2.7	2.2	17

[a] i denotes the step under consideration in the overall reaction n(copper) + albumin → albumin–(copper)$_n$. K_i is the apparent association constant for step i. $\Delta G°_i'$ (pH 4.80) is the apparent standard free energy change for step i and is calculated from K_i. $\Delta H°_i'$ (pH 4.80) is the calorimetric enthalpy change for step i as calculated from the first derivative of eq 4.

Comparison of Binding Properties of Albumin Samples. The binding data for defatted and deionized bovine plasma albumin preparations are shown in Figure 1 along with the data for crystalline albumin and the calculated binding curve. It appears that the binding properties of the albumin were not altered by subjecting crystalline albumin to further purification.

Effect of Acetate Ion Concentration on Binding. Changes in acetate ion concentration were found to have a pronounced effect on the binding of copper to albumin. For example, if 2-ml bags of 2.5% albumin were suspended in 8-ml volumes of 3 mM cupric acetate, $\bar{\nu}$ was 5.7 when the acetate ion was 0.05 M and decreased to 4.7, 2.4 and 2.1 in 0.10, 0.40, and 0.80 M acetate, respectively. To interpret these binding data the concentrations of Cu^{2+}, $CuOAc^+$, and $Cu(OAc)_2$ were estimated from measured association constants (Yatsimirskii and Vasil'ev, 1960; Sillén and Martel, 1964; Sober, 1968). These estimates permitted plots to be made which related $\bar{\nu}$ to Cu^{2+} concentration and the sum of the Cu^{2+} and $CuOAc^+$ concentrations which were then used to predict $\bar{\nu}$'s for systems containing identical amounts of copper but differing amounts of acetate. The agreement between predicted and observed $\bar{\nu}$'s was far better when the plot relating binding to the sum of Cu^{2+} and $CuOAc^+$ was used rather than the plot relating binding to Cu^{2+} alone.

Proton Evolution Caused by Binding. Figure 3 depicts the data obtained from the experiments designed to determine the number of protons evolved from albumin when it reacts with copper in unbuffered solutions. Since no binding studies were performed in unbuffered solutions it was necessary to estimate the values for $\bar{\nu}$ under these conditions. The estimates used in the preparation of Figure 3 were obtained by use of the previously mentioned plot of $\bar{\nu}$ *vs.* the sum of the Cu^{2+} and $CuOAc^+$ concentrations. The estimated $\bar{\nu}$'s were expected to be larger than those observed for similar copper concentrations in buffered solutions and indeed they were found to range from 41% higher (in the most dilute copper solution) to 6% higher (in the most concentrated copper solution) than those observed in acetate buffer of 0.1 ionic strength.

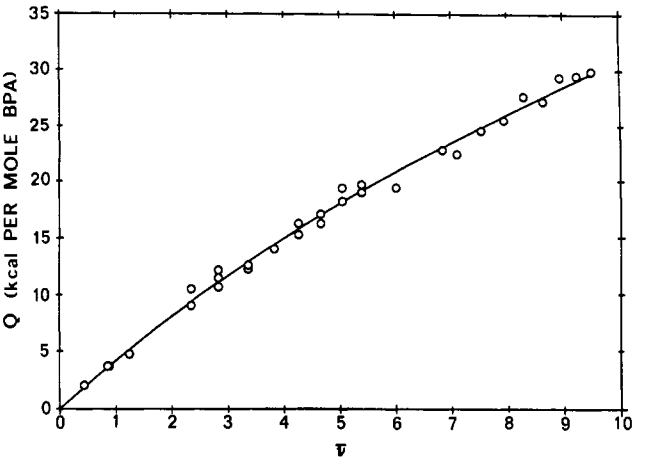

FIGURE 2: Heat absorption (Q) associated with the binding of copper to bovine plasma albumin, expressed as Q *vs.* $\bar{\nu}$. Experimental conditions are analogous to those given in Figure 1. The line is calculated from eq 4 using C_1 = 105 and C_2 = 0.0416. (BPA, bovine plasma albumin.)

During the proton evolution studies, several of the most concentrated solutions initially formed precipitates which dissolved upon further stirring. These cases are indicated by filled circles in Figure 3. In two of these instances the data obtained agreed well with the other data. In the third, how-

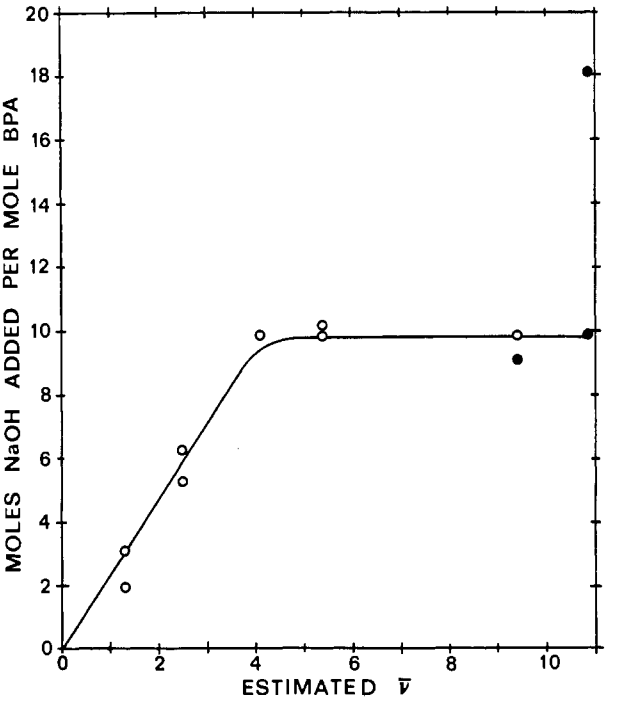

FIGURE 3: Proton evolution associated with the binding of copper to bovine plasma albumin in unbuffered solutions at pH 4.80, expressed as the amount of base required to bring pH back to initial pH (4.80) *vs.* $\bar{\nu}$'s estimated for unbuffered solutions. Albumin concentration 2.5%, 20°. (BPA, bovine plasma albumin.)

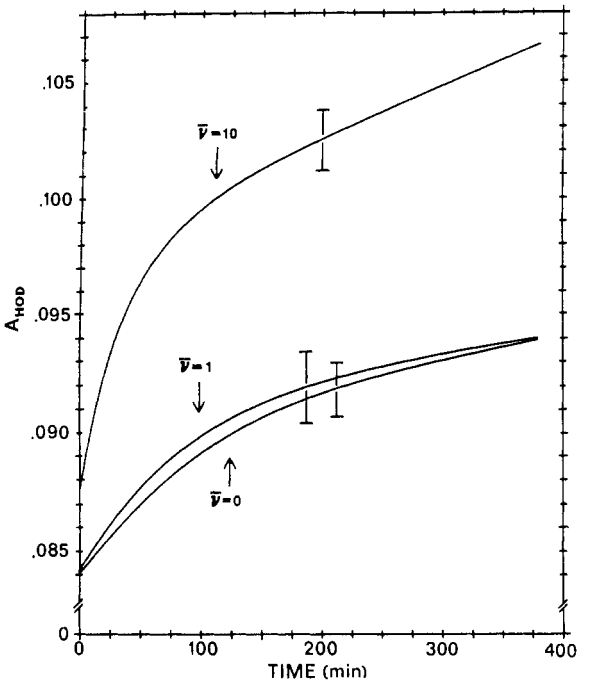

FIGURE 4: Hydrogen-deuterium exchange studies of unbound and copper bound bovine plasma albumin, expressed as the increase in absorbance at 1.41 μ (A_{HOD}) *vs.* time. Solid lines were calculated according to eq 5 and the vertical bars represent corresponding RMS errors. Albumin concentration 2.5% in 5-cm cells, pD 4.8, 20°.

ever, the point obtained was far beyond expected experimental error and was ignored when the curve shown in the figure was drawn. The two linear portions of this curve were obtained by the method of least squares with the two identical points at $\bar{\nu} = 4.1$ being included in both sets of calculations. Examination of the resulting plot suggests that two to three protons are evolved per mol of copper bound during the binding of the first 4 mol of copper and that none are evolved as a result of subsequent copper binding.

Hydrogen–Deuterium Exchange. Approximately 30 readings of the increase in absorbance at 1.41 μ (A_{HOD}) were taken between 10 and 360 min for each run. In total, five runs at $\bar{\nu} = 0$, four at $\bar{\nu} = 1$, two at $\bar{\nu} = 4.4$ and five at $\bar{\nu} = 10$ were performed. Two complete exchange measurements (A_∞) were made for each $\bar{\nu} = 0$ and $\bar{\nu} = 1$, while a single value was obtained for $\bar{\nu} = 10$.

The data exhibited random fluctuations in the initial absorbance reading. This was thought to be due to variable quantities of water in the protein sample and water taken up by the D_2O from the air during dissolution. Therefore, each A_{HOD} *vs.* t measurement for a given experiment was adjusted by a constant factor to make the curves coincide at 360 min. Similarly, the A_∞ readings were scaled by adding the actual change in absorbance between $t = 20$ min and complete exchange to the average A_{HOD} at 20 min obtained after matching the curves at 360 min. For $\bar{\nu} = 0$ and $\bar{\nu} = 1$ the match point was chosen at $A_{HOD} = 0.0938$, while that for $\bar{\nu} = 10$ was $A_{HOD} = 0.1059$. In this way, A_∞ for $\bar{\nu} = 10$ agreed with the average of the four values at $\bar{\nu} = 0$ and 1.

The combined data for a given $\bar{\nu}$ were subjected to computer analysis according to eq 5 (McBride-Warren and Mueller, 1972), where k_1 and k_2 are the rate constants for each of two classes of exchangeable hydrogens while A_1 and A_2 are the corresponding absorbances. In turn, A_1 can be converted to h_i, the number of exchangeable hydrogens in class i, using eq 6, where 0.0534 is the theoretical absorbance change for the 557 peptide hydrogens (Bryan and Nielsen, 1969) of a 2.5% solution of albumin based on an HOD extinction coefficient of 49 cm^2 mol^{-1} (Y. Kakuda and D. D. Mueller, unpublished results). The calculated parameters are presented in Table II and the computed curves in Figure 4. RMS errors were calculated as defined in eq 3 except

$$\sum_{i=1}^{n} w_i$$

was replaced with $n - p$, where n is the number of points and p the number of adjustable parameters.

$$A_{HOD} = A_\infty - A_1 e^{-k_1 t} - A_2 e^{-k_2 t} \quad (5)$$

$$h_i = \frac{A_i}{0.0534} \times 557 \quad (6)$$

TABLE II: Hydrogen–Deuterium Exchange Parameters for Bovine Plasma Albumin and Copper-Bound Albumin at 20°.

$\bar{\nu}$	h_1	k_1 (min^{-1}), $\times 10^2$	h_2	k_2 (min^{-1}), $\times 10^3$	A_∞	RMS Error $\times 10^3$
0	75	0.94	261	0.32	0.1162	1.1
1	77	1.16	250	0.30	0.1155	1.6
10	95	3.39	200	1.81	0.1159	1.4

Clearly, H–D exchange at $\bar{\nu} = 0$ and 1 is indistinguishable within the RMS error. At $\bar{\nu} = 10$, however, approximately 38 additional hydrogens have exchanged at $t = 0$ and 87 more hydrogens reacted over a 6-hr period than for native albumin. In addition, data at $\bar{\nu} = 4.4$, when matched at $A_{HOD} = 0.0938$, show only a slightly greater change over 6 hr than for $\bar{\nu} = 0$ and overlap within the RMS errors given in Table II.

Discussion

Thermodynamic Functions. The calorimetrically determined enthalpy changes for the copper–albumin interaction were found to be positive. Qualitatively this agrees with the temperature-dependance study of this reaction (Klotz and Curme, 1948). Quantitatively, however, the calorimetrically determined enthalpy change for the first bound ion was larger (4.0 *vs.* 2.8 kcal mol^{-1}) and enthalpy changes for successive steps of the reaction were found to decrease rather than increase. These findings point to the need for direct calorimetric measurements of enthalpy changes for complex macromolecular reactions.

The free-energy and entropy changes obtained from our combined use of calorimetric and binding data compared more favorably with those reported by Klotz and Curme (1948). They were similar in both magnitude and sign to those reported earlier and showed decreasing trends as the extent of interaction increased. The rate at which the entropy changes decreased, however, was considerably greater in the present study.

TABLE III: Estimated Change in Net Charge upon Copper Binding.

Copper Species	$\bar{\nu} = 4$			$\bar{\nu} = 10$		
	H^+ Released[a]	OAc^- Bound[b]	Overall Change	H^+ Released[a]	OAc^- Bound[b]	Overall Change
Cu^{2+}	−8 to −10	−1	−1 to −3	−8 to −10	−4	+6 to +8
$CuOAc^+$	−8 to −10	−1	−5 to −7	−8 to −10	−4	−2 to −4

[a] See Figure 3. [b] Based on the data of Teresi and Luck (1952).

One possible explanation for the differences between these and the earlier results could be experimental procedure. Comparison of the experimental conditions reveals slight differences in pH, ionic species present, ionic strength, and temperature. While these could account for some differences in thermodynamic functions, it does not seem reasonable that they could lead to decreasing rather than increasing enthalpy changes as the extent of interaction increased.

Another possible cause for the discrepancies mentioned above could be differences in the purities of the albumin samples used. This was also dismissed for the following reasons. First, it was found that the binding and calorimetric properties of crystalline albumin were not altered upon further purification. Secondly, even if commercial albumin were not subjected to further purification, it can be shown from the data of Hanson and Ballard (1968), Rosseneu-Motreff *et al.* (1970), Sober (1968), and Warner and Weber (1953) that the citrate present as a major contaminant in commercial albumin would not greatly affect the observed enthalpy changes and the small effect would be restricted to the first step of the interaction.

Nature of Copper Species Bound and Proton Evolution. To gain more insight into the nature of the interaction and to check for possible charge effects on the equilibrium, it seemed valuable to determine which copper species were bound and the extent of proton release. Our conclusion that Cu^{2+} and $CuOAc^+$ both bind to albumin not only supports the suggestion of Klotz and Fiess (1951) but extends it to higher ionic strengths. Concomitant proton evolution with interaction of copper with albumin has been reported previously by Fiess and Klotz (1952), Rao and Lal (1958), Breslow (1964), and Peters and Blumenstock (1967). The results from the current study again agree with the earlier observations that the number of protons evolved per mol of copper added decreases with each successive addition, but in addition allow the number of protons evolved to be related to the extent of binding rather than the amount of copper added.

A possible reason for obtaining values of two to three protons released per mol of copper bound might lie in experimental pH errors and/or errors in estimating $\bar{\nu}$ for unbuffered solutions. Alternately, nonintegral proton release could occur if the binding of copper affected the pK of a neighboring ionizable group. Nevertheless, the data indicate that at pH 4.80, protons are released only during the first four steps of the copper–albumin interactions. This again suggests, as did the computer analysis, that the first four steps of the interaction differ from the subsequent steps. Further, depending on their origin, the protons released could make an appreciable contribution to the thermodynamic parameters for the first four steps of the copper–albumin interaction, but not for the subsequent steps of the reaction.

In addition to the above conclusions, the studies of the dependence of $\bar{\nu}$ on acetate ion concentration and the proton evolution associated with the binding of copper allow a further conclusion. One possible explanation for decreased binding constants (K_i) at higher $\bar{\nu}$ values, but constant ionic strength, could be electrostatic repulsion resulting from an increase in net charge on the albumin molecule. To properly estimate the change in net charge, Δz, upon copper binding, three factors need to be considered: (1) proton evolution, (2) the molecular species of copper bound, and (3) the binding of acetate. Table III lists Δz at $\bar{\nu} = 4$ and 10 for each of the two major forms of copper in solution. Earlier it was shown that binding apparently involves both Cu^{2+} and $CuOAc^+$. Therefore, the change in net charge at say, $\bar{\nu} = 10$, would be expected to lie between +8 and −4. If the binding of the two molecular species were equal, then Δz would lie in the range of +1 to +3. Such a small change in Δz at the ionic strengths employed could not account for all the observed decrease in K_i with increased extent of binding.

Finally, the results from these studies indicate that the charge on the copper–albumin complex is not a simple linear function of the extent of interaction.

Conformational Changes. Another factor to consider in a multiple equilibrium process is the possibility of a conformational change. Consequently, H–D exchange measurements were made at several $\bar{\nu}$'s and the results presented in Table II and Figure 4.

Table II lists $h_1 + h_2$ for native albumin as 336. Comparatively, Bryan and Nielsen (1969) using graphical analysis and a cryosublimation exchange technique, reported $h_1 + h_2$ as 302 at pH 4.80 and 30°. Considering the differences in technique and temperature, agreement within 11% appears satisfactory. Their values of k_1 and k_2, however, are just slightly higher than those reported here.

McBride-Warren and Mueller (1972) have shown that in lysozyme virtually all the exchange after 10 min at 25° resulted from peptide N–H. If these conclusions are applicable to albumin, then most, if not all, of the 336 hydrogens would be from the polypeptide backbone. Consequently, it seems unlikely for a conformational change to affect the rates of all the peptide units equally, as would be required for superposition of the $\bar{\nu} = 0$ and $\bar{\nu} = 1$ curves matched at 360 min. Similarly, the $\bar{\nu} = 4.4$ data do not seem to show a significantly different exchange behavior from that of native albumin. Apparently, the first four copper moieties bound do not promote an extensive structural modification in albumin. To the contrary, when $\bar{\nu} = 10$, a substantial alteration in exchange behavior is noted.

One possible explanation for the enhanced exchange could be the acquisition of a net positive charge upon binding Cu^{2+} and/or $CuOAc^+$. Kakuda *et al.* (1971) found pronounced effects on the exchange rate of polymeric amide groups as the net charge varied. It was possible to correct for these effects

using a modified expression for catalysis by deuterium (D^+) and deuteroxide ions (OD^-)

$$k = k_0 + k_D^0(D^+)10^{-2wz} + k_{OD}^0(OD^-)10^{2wz} \quad (7)$$

where k is the observed rate constant for H–D exchange, k_0 is the rate constant for any spontaneous exchange (usually negligible), k_D^0 and k_{OD}^0 the intrinsic acid and base catalytic constants, respectively, w an electrostatic interaction factor obtained from proton titration data, and z the net charge on the macromolecule. (The factor of 2 in the exponent reflects enhanced charge effects noted for amide groups over that predicted from w for carboxyl side chains.) Provided peptide N–H groups behave as those of the polyacrylamide, it should be possible to use eq 7 to predict the change in rate resulting from copper binding. Furthermore, if the pD of the minimum exchange rate for albumin can be assumed to lie below 4, as it does for most proteins (Willumsen, 1971), the reaction would be predominantly base catalyzed at pD 4.8 and a net positive charge would accelerate exchange. In this case the rate enhancement factor will be given by

$$k_m/k = 10^{2w\Delta z} \quad (8)$$

where k_m and k are the rate constants for H–D exchange in the modified and unmodified proteins, respectively, and $\Delta z = z_m - z$.

Referring to Table II, the substitution of $\Delta z = +8$ into eq 8, with $w = 0.022$ (Tanford *et al.*, 1955), gives $k_m = 2.2k$. Comparison of the measured rate constants at $\bar{\nu} = 10$ to those at $\bar{\nu} = 0$ gives $k_{1m} = 3.6k_1$ and $k_{2m} = 5.7k_2$. Therefore, charge effects alone cannot account for all the rate enhancement, even within the maximum possible Δz. Consequently, it is apparent that a conformational change has occurred which resulted in a general loosening of the native structure. The conformational change, however, does not appear to produce new binding sites with greatly different K_i's. This may mean that most, if not all, of the observed binding sites are charged groups which are exposed already in the protein. Interestingly, the proton titration data of Tanford *et al.* (1955) showed that albumin begins to expand at a low positive net charge. Perhaps copper binding mimics protonation by initiating the N–F transition.

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