Thionine absorbs quite strongly and, under the proper conditions, the quantum yield for bleaching approaches unity, so that most of the quanta falling on the system cause reaction. Past this point, however, one may see two possibilities of loss. Some of the energy of the single quantum is converted into the chemical energy of the semiferric pair, but it is not known how much of this is lost and how much is converted. (This question will be discussed more fully in the next paper of this series although at the present concentrations one may estimate a conversion of about 15% of the ~2 e.v. quantum.) A more likely possibility of loss may be found in the electrode process. Since the spontaneous back reaction in the body of the solution competes with the electrode process, it is a bit difficult to understand how *any* significant amount of electrical work may be taken from the system (indeed, this may be the situation; no power measurements have been published for photogalvanic cells). A means of separating the energetic species, of course, would eliminate most of this loss.

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STANFORD, CALIF.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY OF THE UNIVERSITY OF MINNESOTA]

The Interaction of Copper(II) with Bovine Serum Albumin[1]

BY I. M. KOLTHOFF AND B. R. WILLEFORD, JR.[2]

RECEIVED APRIL 28, 1958

Native bovine serum albumin (BSA) can be titrated amperometrically with copper(II) in an ammonia buffer using the rotated platinum wire or rotated dropping mercury electrode as indicator electrodes. At the end-point, one mole of copper is bound per mole of albumin. The reactive group is not sulfhydryl. Nickel(II) forms a stable complex under the same experimental conditions as copper(II) does. BSA denatured in 4 *M* guanidine hydrochloride contains the same reactive group as in the native state. However, an excess of copper oxidizes the sulfhydryl to disulfide whereas this reaction does not occur with native BSA. Sulfhydryl in native BSA can be oxidized by oxygen when one mole or more copper(II) per mole BSA is present. The titration with copper is proposed as a rapid, specific method for the determination of albumin in blood serum.

In work carried out in this Laboratory, it has been found that the sulfhydryl group in cysteine (RSH) is oxidized by copper(II) in ammoniacal medium to cystine (RSSR)[3] according to the overall reaction $2RS^- + 2Cu^{(II)} \rightarrow RSSR + 2Cu^{(I)}$. In the presence of sulfite ion, the reaction is $2Cu^{(II)} + RS^- + SO_3^= \rightarrow 2Cu^{(I)} + RSSO_3^-$. It was of interest to us to determine whether similar reactions occur with the sulfhydryl group in native and denatured bovine serum albumin (BSA).

The copper complexes of a number of proteins including BSA have been investigated by Klotz, *et al.*[4] In particular, it was reported that copper is bound through the albumin sulfhydryl group under the experimental conditions chosen. The formation of complexes between cations and proteins recently has been reviewed by Gurd and Wilcox.[5]

Experimental

Materials.—The bovine serum albumin was obtained from Armour Laboratories, and the guanidine hydrochloride (GHCl) from Eastman. Details of the properties of the BSA, preparation of solutions and purification of the GHCl have been described previously.[6] γ-Globulin was a sample obtained from Professor John T. Edsall of the Harvard University Medical School. Conductivity water was used in the preparation of all solutions. Metal ion solutions were prepared from reagent grade commercial chemicals and analyzed by standard methods. Solutions were deaerated with Linde nitrogen of 99.9% purity. All other chemicals used were reagent grade.

Instrumentation.—Current–voltage curves were measured manually by a circuit similar to that described by Lingane and Kolthoff[7] and automatically with a Leeds and Northrup type E Electrochemograph. Amperometric titrations were carried out using the manual circuit. The platinum electrode was rotated at a constant speed of 600 or 900 r.p.m. by a Bodine synchronous motor. The rotated dropping mercury electrode was of the type A described by Stricks and Kolthoff[8]; a rotation speed of 300 r.p.m. was used. All potentials were measured against the saturated calomel electrode. Measurements of *p*H were made with a Beckman Model H-2 *p*H meter.

Titration Procedure.—The titration vessels were 125-ml. beakers fitted with rubber stoppers with holes for the indicator electrode, nitrogen inlet and outlet, salt bridge and buret. The proper quantity of buffer (usually 25 ml. for R.p.e. titrations, 60 ml. for R.d.m.e) was placed in the titration cell and deaerated with a stream of nitrogen which had been passed previously through buffers of the same composition. The *p*H of the solution after completion of the titration was measured frequently and in no case was there any significant change. When deaeration was complete, the BSA solution (air-free) was introduced and the flow of nitrogen continued for several minutes. The native albumin solutions foamed considerably, and care was taken to avoid any loss of protein through the nitrogen outlet tube. On the other hand, the denatured protein solutions showed very little tendency to foam. In current–voltage curve measurements, the stream of nitrogen was diverted to pass over the surface of the solution, while in amperometric ti-

(1) A preliminary report of this work appeared in THIS JOURNAL, **79**, 2656 (1957).

(2) On leave from Bucknell University, Lewisburg, Pennsylvania.

(3) I. M. Kolthoff and W. Stricks, THIS JOURNAL, **73**, 1728 (1951); *Anal. Chem.*, **23**, 763 (1951).

(4) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948); H. A. Fiess and I. M. Klotz, *ibid.*, **74**, 887 (1952); I. M. Klotz, J. M. Urquhart and H. A. Fiess, *ibid.*, **74**, 5537 (1952); I. M. Klotz, J. M. Urquhart, T. A. Klotz and J. Ayers, *ibid.*, **77**, 1919 (1955); I. M. Klotz, I. L. Faller and J. M. Urquhart, *J. Phys. Colloid Chem.*, **54**, 18 (1950); I. M. Klotz and H. A. Fiess, *ibid.*, **55**, 101 (1951).

(5) F. R. N. Gurd and P. E. Wilcox, *Advances in Protein Chem.*, 11, 311 (1956).

(6) I. M. Kolthoff, A. Anastasi, W. Stricks, B. H. Tan and G. S. Deshmukh, THIS JOURNAL, **79**, 5102 (1957).

(7) J. J. Lingane and I. M. Kolthoff, *ibid.*, **61**, 825 (1939).

(8) W. Stricks and I. M. Kolthoff, *ibid.*, **78**, 2085 (1956).

trations it was passed through the solution continuously. Titrants (10^{-1} to 10^{-2} molar) were added from a Gilmont ultramicroburet of 0.1-ml. total capacity.

Results and Discussion

Current–Voltage Curves.—Figure 1 shows the current–voltage curves of copper(II) at the R.p.e. in a buffer 0.1 *M* in ammonia and 0.1 *M* in ammonium nitrate (curve B). The first wave corresponds to the reduction of copper(II) to copper(I). No copper waves are observed when BSA is present in a molar concentration greater than that of copper (curve D). Curve E illustrates the reappearance of the copper waves when an excess of copper is present. These curves indicate that amperometric titration of BSA with copper(II) should be possible at −0.3 to −0.4 volt. Titration at this potential produces copper(I) and coating of the electrode surface with a metallic film does not occur.

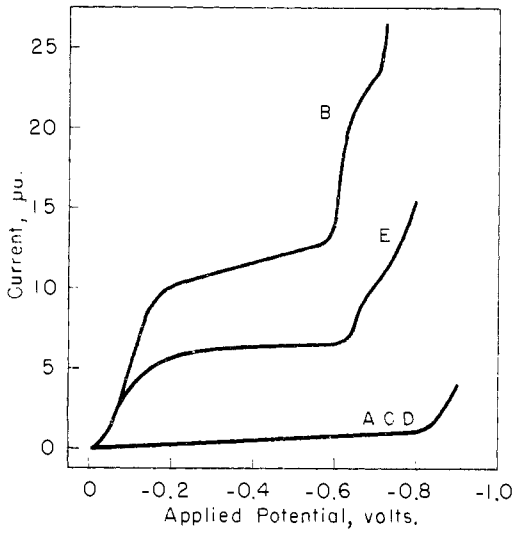

Fig. 1.—Current–voltage curves at R.p.e., 900 r.p.m. 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3, *p*H 9.2: A, residual; B, 4.0×10^{-5} *M* $Cu^{(II)}$; C, 7.3×10^{-5} *M* BSA; D, 7.3×10^{-5} *M* BSA + 6.5×10^{-5} *M* $Cu^{(II)}$; E, 7.3×10^{-5} *M* BSA + 11.0×10^{-5} *M* $Cu^{(II)}$.

Curve A in Fig. 2 shows the current–voltage curve of copper(II) in the presence of 4 *M* potassium chloride. It can be seen that the copper(II) wave is decreased in height and is spread out over a larger potential range than is the case in the absence of chloride. When guanidine hydrochloride (GHCl) is added, the copper(II) wave height is depressed considerably more (curve B). Once again it can be seen that addition of BSA causes the copper waves to disappear (curve C); they reappear when a sufficient excess of copper(II) is present (curve D). In all cases, amperometric titration at −0.4 volt appears feasible.

Amperometric Titrations.—Numerous titrations of native BSA with copper(II) in ammoniacal buffer at *p*H 9.2 indicate a rapid reaction in a mole ratio of copper(II) to BSA of 1.00 ± 0.03. If, after addition of an excess of copper, the solution is allowed to stand, a slower reaction of the excess copper(II) with BSA is indicated by a gradual decrease in the diffusion current. The titrations with copper(II) were continued after various times of standing. In all cases, the slope of the excess reagent line was the same as the original titration line. Extrapolation to the residual current line gives a measure of the total amount of copper(II) reacting (see Fig. 3). When 2 moles of copper(II) per mole of BSA is present, 0.34 additional mole of copper(II) reacts after 1 hr. and 0.57 mole after 21 hr. When four moles of copper(II) is added originally, the additional copper(II) reacting is 0.59 mole and 0.60 mole after 4 and 21 hr., respectively. In all cases the total copper(II) reacting seems to approach 1.60 moles per mole of BSA on long standing under the conditions of these experiments. This slow reaction is not one between copper(II) and sulfhydryl, as is evident from the results in Table I discussed below.

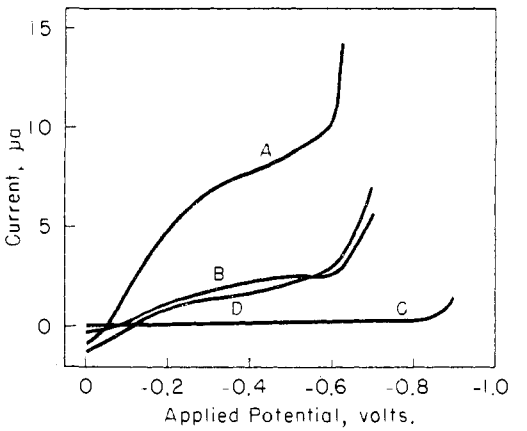

Fig. 2.—Current–voltage curves of $Cu^{(II)}$; R.p.e. at 900 r.p.m.: A, 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3 + 4 *M* KCl, *p*H 9.3, 4×10^{-5} *M* $Cu^{(II)}$; B, 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3 + 4 *M* GHCl, *p*H 9.0, 4×10^{-5} *M* $Cu^{(II)}$; C, 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3 + 4 *M* GHCl, *p*H 9.0, 9.3×10^{-4} mmole BSA, 9.6×10^{-4} mmole $Cu^{(II)}$ (3.8×10^{-5} *M*); D, 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3 + 4 *M* GHCl, *p*H 9.0, 9.3×10^{-4} mmole BSA, 2.4×10^{-3} mmole $Cu^{(II)}$.

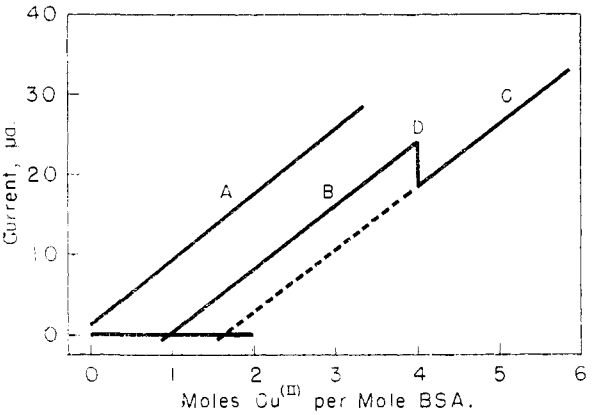

Fig. 3.—Titration of BSA with $Cu^{(II)}$; 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3, *p*H 9.2, 10^{-3} mmole BSA. Time of standing at point D, 21 hr., R.p.e. at 900 r.p.m; A, blank; B, first titration; C, second titration.

The results of a number of titrations of native BSA with copper(II) followed by a second titration with silver(I) or mercury(II) chloride are given in

Table I. A typical titration curve is shown in Fig. 4. It can be seen that after 1 or 2 moles of copper(II) per mole of BSA has been added, 0.68 equivalent of sulfhydryl per mole of BSA is titrated with silver(I) or mercury(II) chloride even after standing with an excess of copper for periods up to 21 hr. The initial rapid reaction of 1 mole of copper(II) per mole of BSA is again indicated. Included also in Table I are the results of two experiments in which 0.68 mole of silver(I) or mercury(II) chloride is added prior to titration with copper(II); this does not prevent the reaction of the copper(II) with the BSA. All these results indicate neither the fast nor the slow reaction of copper(II) with BSA involves the sulfhydryl group.

TABLE I

TITRATION OF 10^{-3} MMOLE BSA IN 25 ML. BUFFER

(0.1 M NH_3 + 0.1 M NH_4NO_3, pH 9.2) with copper(II) and silver(I) or mercury(II) chloride. Applied potential −0.4 volt; R.p.e. at 900 r.p.m.

Moles $Cu^{(II)}$ added per mole BSA	Time of standing,[a] min.	2nd Titrating agent, M	Reaction ratio Cu/BSA	Reaction ratio M/BSA
1.0	5	$Ag^{(I)}$	(1.00)	0.67
1.0	15	$Ag^{(I)}$	(1.00)	.63
2.0	5	$Ag^{(I)}$	0.97	.67
2.0	21 hr.	$Ag^{(I)}$	..	.66
2.0	5	$HgCl_2$	.99	.70
..[c]	5	$Ag^{(I)}$	.92[b]	(.68)
..[d]	5	$HgCl_2$	.98	(.68)

[a] Time between end of 1st titration and start of 2nd titration. [b] Low result attributed to change in platinum electrode surface caused by plating out of silver. [c] Titration with $Cu^{(II)}$ *after* addition of 0.68 mole of $Ag^{(I)}$ per mole of BSA. [d] Titration with $Cu^{(II)}$ *after* addition of 0.68 mole of $HgCl_2$ per mole of BSA.

Effect of Oxygen.—The sulfhydryl group in native BSA is not oxidized by oxygen in ammoniacal buffer at pH 9.[6] However, when solutions of BSA at pH 9 which contain an excess of copper(II) are exposed to air, the amount of titratable sulfhydryl decreases significantly. For example, after 1, 3 and 21 hr., the sulfhydryl values were 0.41, 0.35 and 0.16, respectively. Experiments in which 10^{-3} mmole BSA in ammoniacal buffer of pH 9 and containing varying amounts of copper(II) was saturated with a stream of oxygen for 20–30 minutes, allowed to stand overnight in a closed vessel and then titrated with silver(I) after removal of the oxygen revealed that with 0.75 mole of copper(II) or less per mole BSA all the sulfhydryl was titrated even after 25 hr., while no sulfhydryl was found after this period of time with 1.0 or more moles of copper per mole of albumin. In a similar experiment with 2.0 moles of copper(II) per mole of BSA saturated with nitrogen instead of oxygen, all the sulfhydryl was found. Since only the excess copper(II) can promote the oxidation of the sulfhydryl groups, the previously reported observation that a trace of copper(II) has no effect on the rate of oxidation of BSA while it greatly accelerates the oxidation of cysteine to cystine[6] is readily explained.

Effect of pH and Buffer Composition.—Current–voltage curves of copper(II) solutions in buffers of total ammonia concentration to 2 M both in the presence and absence of BSA indicate that amperometric titrations of copper(II) at −0.4 volt in these media are feasible though the copper waves are somewhat ill defined at the higher buffer concentrations. No titration in 0.01 M ammonia at pH 8 was possible, for a curved excess reagent line was obtained. A stoichiometric reaction between copper(II) and BSA was found in the pH range 8–10 in buffers with ammonia concentrations ranging between 0.03 and 0.5 M and ammonium nitrate concentrations varying between 0.01 and 1 M.

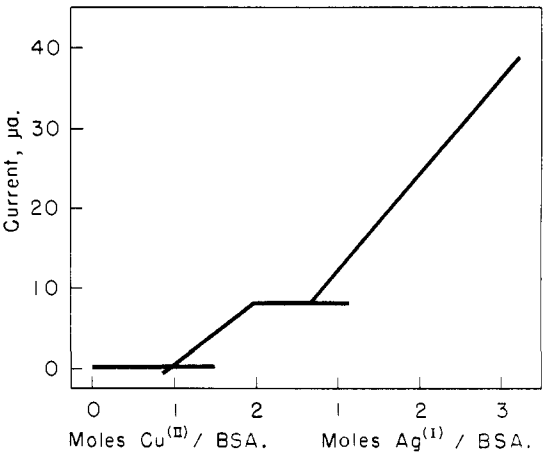

Fig. 4.—Titration of BSA with $Cu^{(II)}$ followed by titration by $Ag^{(I)}$: 10^{-3} mmole BSA in 25 ml. buffer, 0.1 M NH_3 + 0.1 M NH_4NO_3, pH 9.2, R.p.e. at 900 r.p.m.

Titrations of Denatured BSA.—As mentioned previously, current–voltage curves in the presence of 4 M GHCl (Fig. 2) indicate that amperometric titrations of BSA with copper(II) at −0.4 volt should be feasible even though the diffusion currents observed are not large. A number of such titrations indicate that there is a rapid reaction of 1.3 moles of copper(II) with denatured BSA. This is followed by an indefinite slower reaction (more rapid however than the secondary reaction with native BSA) which depends upon both the amount of excess copper(II) present and the time of standing. There is no apparent pattern to this further slow reaction. In a typical case, the first excess reagent line gave a Cu/BSA reaction ratio of 1.31. When the ratio of copper(II) added to BSA present reached 4, the solution was allowed to stand for 20 minutes, during which time the diffusion current dropped, at first rapidly, then more slowly, from 5.0 to 3.2 μa. More copper(II) was then added. Extrapolation of this excess reagent line gave a Cu/BSA ratio of 2.36. Again, on standing for 20 minutes (copper(II) added to BSA ratio of about 7) the diffusion current dropped from 8.8 to 7.8 μa. Further addition of copper(II) yielded an excess reagent line which extrapolates to a Cu/BSA reaction ratio of 3.05. Further uptake of copper(II) was indicated by continued decrease of the diffusion current. This slow but continuous copper(II) consumption is attributed to a slow hydrolytic fission of the disulfide groups in the albumin, the sulfhydryl formed being oxidized by copper(II). Copper(II), like silver(I) and mercury(II), promotes this hydrolytic fission.

A series of experiments was carried out in which varying amounts of copper(II) were added to the protein prior to denaturation and then an amount of air-free solution of GHCl in buffer to give a final concentration of GHCl of 4 *M* was added. Addition of the GHCl solution caused the diffusion current of the excess copper(II) to drop rapidly. When the diffusion current reached a steady value (usually after about 8 minutes), titration with copper(II) was then resumed, and the excess reagent line extrapolated to the residual current line. Figure 5 shows one of these titrations. The results confirm the increase of Cu/BSA reaction ratio from 1.0 to 1.3 when the protein is denatured in 4 *M* GHCl.

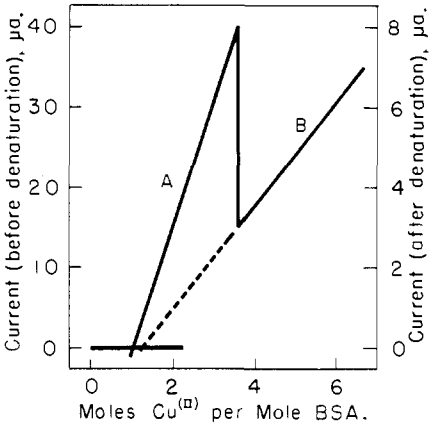

Fig. 5.—Titration of BSA with $Cu^{(II)}$ before and after denaturation; R.p.e. at 900 r.p.m.: 10^{-3} mmole BSA; A, 0.1 *M* NH_3 at 0.1 *M* NH_4NO_3; B, 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3 + 4 *M* GHCl.

A series of titrations was carried out in which the BSA was treated with 0.67 mole of silver(I) or mercury(II) chloride prior to denaturation in 4 *M* GHCl. After five minutes denaturation, the solution was titrated with copper(II). BSA treated with silver(I) gave normal titration lines indicating reaction of one mole of copper(II) per mole of BSA. Titrations of the mercury(II) chloride treated BSA were less satisfactory because the excess reagent lines were curved. However, they too indicate a drop in the Cu/BSA reaction ratio from the 1.3 observed with untreated denatured BSA to somewhat less than 1.0. The silver(I) or mercury(II) chloride ties up the sulfhydryl group in the denatured protein and renders it unavailable for reaction with copper(II). Confirmatory evidence for this was obtained from an experiment in which the denatured protein was treated with ferricyanide. It has been found that the sulfhydryl group of native BSA is not oxidized by ferricyanide but that denaturation of the protein renders the sulfhydryl susceptible to rapid ferricyanide oxidation.[9] A sample of BSA in denaturing mixture was treated with ferricyanide and then titrated with copper(II); a Cu/BSA reaction ratio of 1.01 was found.

On the basis of these experiments, it is suggested that the sulfhydryl group is involved in the rapid reaction of 1.3 moles of copper(II) per mole of denatured albumin. The series of reactions (1), (2), (3) and (4), similar to those proposed for the oxidation of cysteine by copper(II),[3] is proposed.

$$2Cu^{(II)} + 2PSH \longrightarrow 2Cu^{(I)} + 2PS + 2H^+ \quad (1)$$

$$2PS \longrightarrow PSSP \quad (2)$$

$$2Cu^{(I)} + 2PSH \longrightarrow 2PSCu^{(I)} + 2H^+ \quad (3)$$

$$2Cu^{(II)} + 4PSH \longrightarrow PSSP + 2PSCu^{(I)} + 4H^+ \quad (4)$$

One mole of albumin contains 0.67 mole of sulfhydryl which, according to equation 4, reacts with 0.34 mole of copper(II). Presumably 1.0 mole of copper reacts at the same site as in native BSA. Thus, the total copper consumption should be 1.34 moles per mole of denatured albumin, in good agreement with the average experimental value of 1.31. This interpretation is supported by viscosity measurements carried out by B. H. Tan in this Laboratory. The reduced viscosity of 1% albumin in 4 *M* GHCl in a buffer of *p*H 9 was found to be 0.19; after oxidation with 2 equivalents of ferricyanide (per sulfhydryl) or of 4 moles of copper(II), the reduced viscosity increased to 0.28 as a result of dimer formation.

Effect of Other Metal Ions.—Titrations of 10^{-3} mmole of BSA by copper(II) in the presence of varying amounts of cobalt(II), nickel(II) and zinc(II) ions have been carried out. Results are given in Table II. It can be seen that nickel(II) and cobalt(II) are effective in suppressing the reaction of copper(II) with native BSA while zinc(II), even in very large excess, has practically no effect.

TABLE II

EFFECT OF $Co^{(II)}$, $Ni^{(II)}$ AND $Zn^{(II)}$ ON REACTION OF $Cu^{(II)}$ WITH NATIVE BSA

25 ml. buffer (0.1 *M* NH_3 + 0.1 *M* NH_4NO_3, *p*H 9.2); 10^{-3} mmole BSA (concn. = 4×10^{-5} *M*); R.p.e. at 900 r.p.m., −0.4 volt *vs.* SCE.

Metal	Moles M/mole BSA	Cu/BSA reaction ratio
(Blank)	0	1.02, 1.00, 0.99
Co	10	0.51
Co	3	.53
Co	2	.64
Co	1	.92
Ni	44	.00
Ni	5	.07
Ni	3	.05
Ni	2	.24
Ni	1	.36
Zn	47	.91

The presence of 3 or more moles of nickel(II) per mole of BSA reduces the copper(II)-BSA reaction to less than 10% of its value in the absence of nickel(II); lesser amounts of nickel(II) permit more copper(II) reaction. Cobalt(II) is less effective than nickel(II) in suppressing the copper(II) reaction. Presumably the nickel(II) and to a lesser extent cobalt(II) prevent the reactions of copper(II) by blocking the site on the BSA molecules where the copper(II) reaction takes place. The order of stabilities of complexes of these metals is Co < Ni < Cu.[10] In agreement with this order nickel(II) was found to be more effective than cobalt(II) in preventing complex formation with copper(II).

(9) I. M. Kolthoff and A. Anastasi, THIS JOURNAL, **80**, 4248 (1958).

(10) H. Irving and R. J. P. Williams, *J. Chem. Soc.*, 3192 (1953).

Experiments with the Rotated Dropping Mercury Electrode (R.d.m.e.).—In order to achieve the more negative potentials necessary to investigate further the effect of other metals, a number of experiments were carried out using the rotated dropping mercury electrode[8] as the indicator electrode. Current–voltage curves of the metal ions in the presence of 0.01% polyacrylamide (PAA) as maximum suppressor[11] are shown in Fig. 6. Similar experiments were run in a 1% albumin solution. In 1% BSA solution, the copper(II) and copper(I) waves occur at the same potentials as in the supporting electrolyte with 0.01% PAA in the absence of albumin. The nickel(II) wave is stretched out and shifted to a slightly more positive potential with BSA. The cobalt(II) and zinc(II) waves in the presence of BSA are shifted about 0.1 volt to more positive potentials. The cobalt(II) wave shift also has been observed by Brdicka[12] with the conventional dropping mercury electrode. In the albumin solutions another wave appears after the cobalt and nickel waves near −1.3 volt; this is apparently the protein catalytic wave discussed by Brdicka.[12] It should be noted that in the presence of BSA the cobalt and zinc waves appear when even a small amount of these metals has been added, while with nickel and copper, the molar concentration of metal ion added must exceed that of the BSA before the metal reduction waves appear. This is in accord with amperometric titration data.

Amperometric titrations of BSA with copper(II) in ammoniacal medium at the R.d.m.e. gives the expected Cu/BSA reaction ratio of 1.0. Titration with nickel(II) at −1.15 volt indicates a similar value for the Ni/BSA reaction ratio. Furthermore, addition of exactly 1 mole of copper(II) per mole of BSA prior to titration with nickel(II) effectively prevents the reaction of nickel(II) with BSA. This, along with the previously mentioned fact that nickel(II) suppresses the reaction of copper(II) with BSA as determined at the R.p.e., is additional evidence that both nickel(II) and copper(II) react at the same site on the BSA molecule. On the other hand, titration of BSA with cobalt(II) and zinc(II) gave no indication of any reaction. Apparently, the cobalt–albumin complex is much less stable than the copper(II) complex.

In order to investigate the effect of denaturing the protein on the nickel(II)–BSA reaction, current–voltage curves of nickel(II) in a medium 0.1 *M* in ammonia, 0.1 *M* in ammonium nitrate and 4 *M* in GHCl were determined. No nickel waves were observed, and so titration in this medium is precluded.

Reactive Group in BSA.—It has not as yet been possible to identify the group in albumin which is reactive toward copper(II) and nickel(II). A sample of BSA was hydrolyzed to break down the peptide chain; titration of the amino acid residues with copper(II) gave no sign of significant reaction. Titration of pure aspartic acid similarly was not possible. These results indicate that some structural feature of the BSA molecule is responsible for the binding of these metals; this structural feature apparently is destroyed by hydrolysis. The reactive group must be weakly acidic in character, for at *p*H 4.7 in acetate buffer, current–voltage curves at the R.d.m.e. of copper(II) in the presence of BSA gave no indication of any reaction. It also appears that, although BSA is heterogeneous with respect to sulfhydryl, being composed of $^2/_3$ mercaptalbumin and $^1/_3$ sulfhydryl-free albumin, the two kinds of albumin have the same property as far as copper binding is concerned.

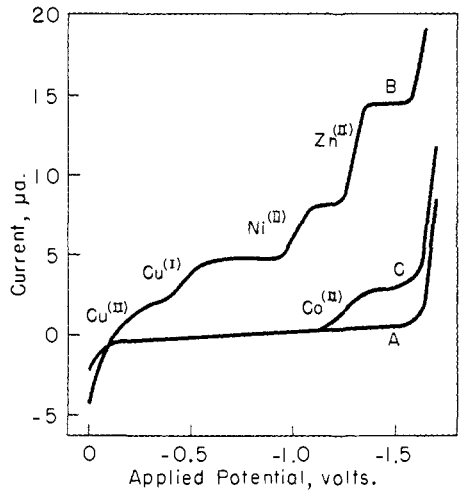

Fig. 6.—Current–voltage curves of metal ions: 0.1 *M* NH_3 + 0.1 *M* NH_4NO_3 + 0.01% PAA, *p*H 9.2, R.d.m.e. at 300 r.p.m: A, residual; B, 4.8×10^{-5} *M* $Cu^{(II)}$ + 4.7×10^{-5} *M* $Ni^{(II)}$ + 8.3×10^{-5} *M* $Zn^{(II)}$; C, 5×10^{-5} *M* $Co^{(II)}$.

Titrations of Blood Serum.—An attempt was made to titrate human γ-globulin with copper(II) in ammonia buffer at *p*H 9.2, but no reaction was found. The fact that copper(II) does not react with human γ-globulin, but does react stoichiometrically with BSA, suggests the possibility that the reaction may be used for the determination of albumin in blood serum. In order to show that under these recommended conditions albumin is the only protein in human blood serum which reacts with copper(II), a sample of fractionated blood serum, made available to us by Dr. H. H. Zinneman of the Minneapolis Veteran's Hospital, was titrated under the usual conditions. This sample had been fractionated by the starch block electrophoresis technique. The total protein of each fraction and of the whole serum was determined by the tyrosine method. Satisfactory agreement was obtained between the value for the whole serum and the sum of the values for each of the fractions. In addition, a Kjeldahl determination of the nitrogen content of the whole serum was made, and from this the total protein in the whole serum was calculated assuming a value of 16% nitrogen.[13] Since the tyrosine values tend to be slightly low, the protein content of each fraction was corrected so that agreement with the Kjeldahl

(11) I. M. Kolthoff, Y. Okinaka and T. Fujinaga, *Anal. Chim. Acta*, **18**, 295 (1958).

(12) R. Brdicka, *Collection Czechoslov. Chem. Communs.*, **5**, 112, 148, 238 (1933); *Biochem. Z.*, **272**, 104 (1934).

(13) F. Haurowitz, "Chemistry and Biology of Proteins," Academic Press, Inc., New York, N. Y., 1950, p. 12.

TABLE III

TITRATION OF BLOOD SERUM WITH $Cu^{(II)}$

Medium—0.1 M NH_3 + 0.1 M NH_4NO_3, pH 9.2; R.p.e. at 600 r.p.m., −0.4 volt.

Serum fraction	$Cu^{(II)}$ value, as g. % albumin
Albumin	3.41
α_1-globulin	0.25
α_2-globulin	0
β-globulin	0
γ-globulin	0
Total	3.66
Whole serum, by $Cu^{(II)}$	3.68
Whole serum, tyrosine (cor.)	3.70

value for the whole serum was obtained. Results of copper(II) titrations on both whole serum and the fractions are given in Table III. While the unusually close agreement between values is certainly fortuitous, it is significant that over 93% of the copper titer of the whole serum is accounted for in the albumin fraction. The remaining 6% occurs in the α_1-globulin fraction and can be attributed to overlap of the albumin and α_1-globulin fractions.

The amperometric copper titration now is being applied to the rapid and specific determination of albumin in blood serum. Results will be reported elsewhere.

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Some Metal Complexes of Glycine Peptides[1a,b]

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The formation constants of Cd(II), Ni(II) and Zn(II) complexes of glycylglycine, glycylglycylglycine, tetraglycine and glycine amide have been determined. With each of these three metal cations, the glycine amide and the three peptide complexes are about equally stable. With Mg(II) cation, the glycylglycine and tetraglycine complexes are equally stable. It is shown that the three glycine peptides probably have common coördination sites, which are probably the terminal amino group and the immediately adjacent peptide group.

Introduction

Evans and Monk[2] reported the formation constants of several metal complexes of glycylglycine and glycylglycylglycine, and suggested that the metal-peptide bonds do not involve the terminal amino and charged carboxylate groups. More recently, Li, *et al.*,[3] obtained the formation constants of Co(II) complexes of glycylglycine, glycylglycylglycine and tetraglycine, using the methods of pH and ion exchange, and found that these three glycine peptides are about equally stable. They postulated that the three glycine peptides probably have common coördination sites toward Co(II), and that the sites are probably the terminal amino group and the immediately adjacent peptide group. It is of interest to continue the study of the peptide complexes with other metal ions, and this paper presents the results on the Cd(II), Zn(II), Ni(II) and Mg(II) complexes.

Experimental

Materials.—The glycine peptides and glycine amide were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Stock solutions of metal salts were prepared and analyzed by conventional means. All chemicals were of C.P. grade.

Procedure.—Measurements of pH were made with a Beckman Model G pH meter with external electrodes. Only freshly prepared solutions of the peptides were used, and nitrogen gas was bubbled through the solution. The instrument was standardized against Beckman standard buffer solutions, pH 4 and 7.

Polarographic current–voltage curves were made manually with a Fisher Elecdropode. All potentials were measured against a saturated calomel electrode (S.C.E.) and the half-wave potentials were corrected for the IR drop.

Results

(A) Titration of 1:1 (Peptide:Metal Ion) Molar Mixtures.—The formation constants of the 1:1 complexes have been determined by pH titration of solutions containing 0.01 M glycine peptide or glycine amide, 0.01 M metal(II) nitrate and 0.12 M KNO_3. As examples, the titration data for the Ni(II) complex of tetraglycinate, and the Cd(II) complex of glycylglycylglycinate at 25° are given in Table I. The symbols (A^-) and $\bar{n}$ represent the total concentration of free peptide anion in solution and the average number of moles of peptide anion bound per mole of the divalent metal ion, respectively.

Values of k_1 are calculated for data in the region $\bar{n}<0.5$ only, because at higher $\bar{n}$ values, protons from the amide groups in the peptides are titrated. A summary of the formation constants of the 1:1 complexes of Cd(II), Zn(II), Ni(II) is given in Table II. Our values of log k_1 (u = 0.15) of the glycylglycinate and glycylglycylglycinate complexes are in general about 0.3 log unit lower than the corresponding values listed by Evans and Monk[2] at u = 0. Part of the difference probably is due to the difference in ionic strength. No value for the tetraglycinate complex of these metal cations has been reported in the literature.

(B) Titration of 1–10 (Peptide–Metal Ion) Molar Mixtures.—The formation constants of the

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(2) W. P. Evans and C. B. Monk, *Trans. Faraday Soc.*, **51**, 1244 (1955).

(3) N. C. Li, E. Doody and J. M. White, THIS JOURNAL, **79**, 5859 (1957).