[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

Slow Intramolecular Changes in Copper Complexes of Serum Albumin. The Role of Neighboring Groups in Protein Reactions

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Slow changes in the spectra and properties of copper complexes of bovine albumin have been shown to be due to metal-catalyzed reactions of an S–S linkage about 3.4 Å. from the sulfhydryl group of the protein.

Introduction

The uptake of a variety of metal ions by proteins appears to be a reversible phenomenon by simple mass-law tests.[1,2] Nevertheless, there are frequent indications that removal of the metal does not return the protein to its initial state.[3,4] The molecular nature of any irreversible changes produced by the bound metal are still obscure, however.

It has been found recently[5] that the binding of cupric ions by bovine serum albumin produces a characteristic absorption band at 375 mμ. By a variety of experiments it can be demonstrated that this band arises from a copper–sulfhydryl bond. With this "indicator" band, further studies have now shown that the sulfide-bound copper is a particularly sensitive site and excites irreversible structural changes in the protein. The molecular nature of these changes has been elucidated, at least in part, and they illustrate the important role played by neighboring groups in providing a pathway for denaturation by metals.

Experimental

Absorption Spectra.—The absorption of light was measured with the Beckman spectrophotometer, model DU, at approximately 25°. One-cm. cells were used. Extinction coefficients, ϵ, were calculated from the equation

$$\epsilon = \frac{1}{cd} \log_{10} I_0/I$$

where I_0 is the intensity of the light emerging from the solvent, I the intensity of the light emerging from the solution, c the total molar concentration of the cupric ion and d the thickness of the absorption cell in centimeters. The solvent or blank solution contained the same concentration of protein as did the copper-containing solution, so that the scattering of light by the protein was automatically compensated. With the 2% protein solutions generally used, this correction is appreciable, particularly in the near ultraviolet.

For the examination of spectra in the absence of air, a Thunberg tube was modified by attachment of a one-cm. absorption cell at the bottom. In some experiments, the tube was filled with copper solution and the side-arm with protein, the assembly was alternately evacuated and filled with nitrogen or carbon dioxide, and the solutions were mixed by tipping the tube. In other experiments the modified Thunberg tube was made the third member of a train, the first two sections of which contained solutions of copper ion and protein, respectively. The entire system was freed of air by permitting a stream of nitrogen to bubble through, the solutions were mixed and transferred under small pressure of nitrogen to the Thunberg tube, which was then closed off and transferred to the spectrophotometer. The cover of the cell compartment of the latter was replaced by a small cardboard box, covered with dull black tape and shaped to fit snugly over the top. In this manner leakage of light into the spectrophotometer was avoided completely.

Ultracentrifugation.—Sedimentation measurements were made with the Spinco ultracentrifuge, model E, operated at 59,780 r.p.m. Two per cent. protein solutions in 0.2 *M* acetate buffer at *p*H 6 were used in all cases. The temperature was 22 ± 1°. These experiments were carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts. We are indebted to Dr. L. R. Hyde for his assistance.

Reagents.—Bovine fibrinogen and crystallized bovine serum albumin, ovalbumin, pepsin and β-lactoglobulin were purchased from Armour and Company. Two samples of human serum albumin were used, one a crystallized preparation (Decanol 10) obtained through the courtesy of Dr. W. L. Hughes, Jr., and the other a sample fractionated from contaminated plasma, 97–99% pure, a gift from Drs. P. H. Bell and R. O. Roblin, Jr. Bovine serum albumin was acetylated according to the procedure of Fraenkel-Conrat, Bean and Lineweaver.[6] Although this particular sample was not analyzed for free amino groups, titrations[7] of closely analogous preparations showed that 60 ± 10% of these groups had been acetylated. Iodinated bovine albumin was prepared by Dr. W. L. Riedeman, following the procedure of Hughes and Straessle.[8] Absorption of light of 312 mμ by this protein corresponded to 6.5 diiodotyrosine residues.

Thioglycerol was obtained as a 54% aqueous solution from Evans Chemetics, Inc. Glutathione was purchased from Schwarz Laboratories, mercaptosuccinic acid from Matheson Co., L-thiolhistidine from the California Foundation for Biochemical Research, and imidazole from Edcan Laboratories. Salyrganic acid was a product of Winthrop-Stearns. The sodium borohydride was a gift of Dr. Walter Nudenberg. The amino acids and other organic chemicals were of highest purity commercially available. Inorganic substances were of reagent grade. The dry nitrogen was a specially purified grade warranted by the manufacturer to contain less than 0.01% impurities (mostly oxygen, carbon dioxide and moisture).

Results and Discussion

In aqueous solutions, under a variety of conditions to be described, an absorption band appears at 375 mμ (as well as in the visible region) when bovine serum albumin is added to cupric ions (Fig. 1). That a copper–sulfhydryl bond is involved was indicated by previous experiments[5] with a number of reagents which block sulfhydryl groups: mercuric ion[9]; an organic mercurial,

(1) I. M. Klotz and H. G. Curme, THIS JOURNAL, **70**, 939 (1948).

(2) F. R. N. Gurd and D. S. Goodman, *ibid.*, **74**, 670 (1952).

(3) W. L. Hughes, Jr., *Cold Spring Harbor Symposia on Quantitative Biology*, **14**, 79 (1950).

(4) F. R. N. Gurd, *J. Phys. Chem.*, **58**, 788 (1954).

(5) I. M. Klotz, J. M. Urquhart and H. A. Fiess, THIS JOURNAL, **74**, 5537 (1952).

(6) H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, **177**, 385 (1945).

(7) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 235 (1936).

(8) W. L. Hughes, Jr., and R. Straessle, THIS JOURNAL, **72**, 452 (1950).

(9) (a) The order of addition of Hg^{++} or Cu^{++} (0.0033 *M*) to the albumin (0.0003 *M*) has no effect on the final outcome. Whether Hg^{++} precedes or succeeds the copper, the 375 mμ peak does not appear at 0.0003 *M* Hg^{++} and is reduced to the same extent at 0.0001 *M* Hg^{++}. It is evident, therefore, that the P–S^- group still exists even after the bond is formed with copper; if the cupric ion oxidized the sulfhydryl in giving rise to the 375 mμ absorption, we should expect the mercuric ion to be no longer capable of displacing copper. Likewise, it was shown that cuprous ion is not responsible for the 375 mμ peak, by overt addition (in an atmosphere of nitrogen) of

salyrganic acid; silver ion. Confirmation of this assignment now is also available from experiments with iodinated and acetylated albumin. In the former protein the –SH group is oxidized and in the latter blocked by acetyl groups. With both proteins, added cupric ions gave no indication of a peak at 375 mμ. Likewise exposure of albumin to less than equimolar hydrogen peroxide markedly decreases the absorption at 375 mμ.

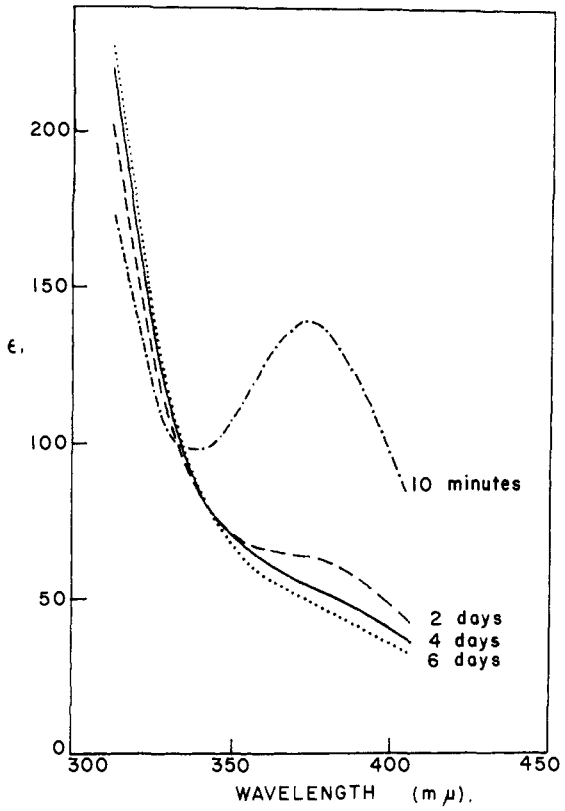


Fig. 1.—Change of absorption band of copper-bovine albumin with time.

The intensity of this peak drops with time, although at *p*H's near 6 the change is so slow that it could be overlooked. A typical example is illustrated in Fig. 1. Over a period of days, the 375 mμ peak drops to a small fraction of its initial value. On the other hand, the drop in absorption near 700 mμ over the same period is only 10–15%.

Molecularity of the Reaction.—The first question one might ask with regard to the molecular basis of these optical changes is whether the change in copper spectrum with time is due to a reaction with groups on the same protein molecule to which the metal is bound or with groups on another protein molecule. At first glance, the latter alternative might seem very likely from the known origin of the 375 mμ band. Since copper is bonded to the –SH of albumin, one might expect, by analogy with the reaction with mercury,[8] dimerization to occur

$$\text{P—S—Cu} + \text{HS—P} = \text{P—S—Cu—S—P} \quad (1)$$

If reaction (1) were to occur, the dimeric species should be readily visible in the ultracentrifuge. Photographs of the schlieren patterns were taken, therefore, over periods up to 12 days, of bovine albumin with copper, as well as of protein alone in the same buffer. No new peaks were observed in the sedimentation patterns (Fig. 2) and the rate of sedimentation of protein in the presence of copper was not detectably different from that in its absence. Thus slow dimerization of albumin through a copper bridge is not responsible for the disappearance of the 375 mμ absorption.

The ultracentrifugal experiments also rule out the reaction

$$\text{P—S—Cu} + \text{Cu—S—P} = \text{P—S—S—P} + 2\text{Cu(I)} \quad (2)$$

which might have been a possibility[10] had any evidence been obtained for a dimeric protein molecule.

Non-involvement of Oyxgen.—Since the optical changes are not due to interactions between two protein molecules, they must relate to some reaction of the protein with another component of the solution or some slow change within the protein molecule itself. Since the copper is bound to the sulfhydryl group of albumin, a likely reactant in the solvent seemed to be dissolved oxygen, which could oxidize the mercapto group. Several methods were used to remove dissolved oxygen: (1) evacuation of air above solution; (2) successive replacements of air by nitrogen; (3) successive replacements of air by carbon dioxide; (4) removal of dissolved oxygen from solutions (before mixing) by slow bubbling of prepurified nitrogen. In no case was any significant change found for the rate of decline of the 375 mμ peak, as compared to the reaction in the presence of air. The results of the last type of experiment are shown in Fig. 3. It is evident, therefore, that reaction with oxygen is not responsible for the decline of the 375 mμ peak.

Dependence on *p*H and Added Reagents.—Some hint as to the mechanism of the disappearance of the 375 mμ peak was obtained from an examination of *p*H effects. It is necessary to proceed with caution, however, since two opposing effects are observed. Increase in *p*H raises the initial height of the peak, as is shown in Fig. 4, until about *p*H 8.[11] Thereafter the height *apparently* drops again, but this behavior may be in part an artifact due to the second effect of increased *p*H, a marked increase in the rate of disappearance of the peak. Data on the kinetics of this reaction at *p*H's 6, 7 and 9 are illustrated in Fig. 3. The results at *p*H's 6 and 7 are direct readings at each *p*H. At *p*H 9, however, the optical absorption by copper ions bound to groups other than the sulfhydryl is so great that it obscures the 375 mμ peak. Consequently the rate of disappearance at this high *p*H was followed

3×10^{-4} *M* cuprous chloride, to a solution containing 3×10^{-4} *M* protein and 1.5×10^{-3} *M* cupric ion; no significant increase in optical density at 375 mμ was observed over that of the solution without added cuprous ion. (b) In another experiment, bovine serum albumin was exposed to (equimolar) iodoacetamide in acetate buffer, *p*H 6.0, for 21 hours at room temperature. A drop of about 50% in the height of the peak was observed. The reaction of iodoacetamide with the mercaptan group of albumin is thus slow and non-selective.

(10) R. Straessle, THIS JOURNAL, **76**, 3138 (1954), has reported that a disulfide-bridged dimer can be prepared by oxidation of the mercaptide with iodine.

(11) This rise in height is probably due to the favorable effect of base on the reaction

$$\text{P—SH} + \text{Cu(II)} = \text{P—S—Cu} + \text{H}^+$$

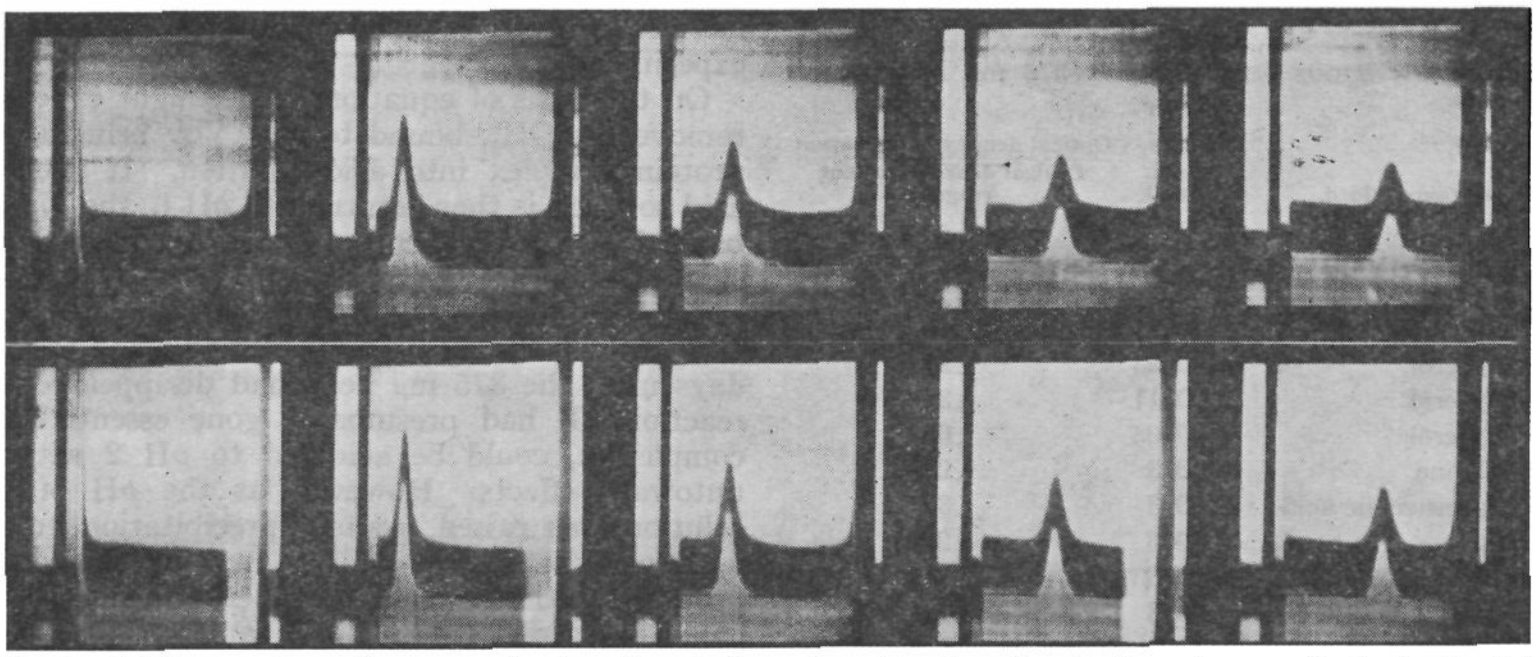


Fig. 2.—Schlieren patterns for copper–bovine albumin (upper) and bovine albumin (lower), respectively, in the Spinco ultracentrifuge. In both cases the proteins were 2% solutions in 0.2 *M* acetate at *p*H 6. The copper concentration was 0.0033 *M*. The times of the photographs are 0, 32, 64, 96 and 128 minutes after attainment of full speed. The bar angle was set at 55° in the upper photograph, at 45° in the lower. The copper–albumin solution had been standing at room temperature for nine days; the 375 mμ band had disappeared completely.

by exposing the copper–albumin solution for a fixed period, adjusting the *p*H back to 6 and reading the optical density. A typical set of such values is included in Fig. 3. A control run in which protein was exposed to the higher *p*H without copper being present and then returned to *p*H 6 gave results just like protein maintained at *p*H 6.

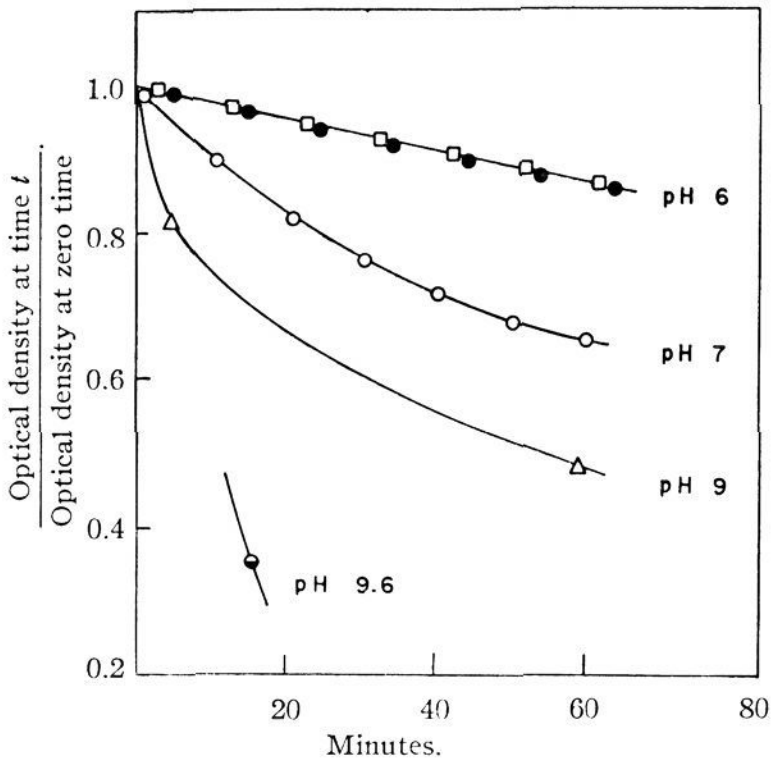


Fig. 3.—Effect of removal of oxygen and of variation in *p*H on rate of disappearance of 375 mμ band: □, in absence of air; all others in presence of air.

These rather striking effects of increased hydroxyl ion concentration suggested the following reaction

$$\text{P—S—Cu} + \text{OH}^- \longrightarrow \text{P—S—Cu—OH} \quad (3)^{12}$$

since it has been shown recently[13] that metals such as copper may act as bridges between proteins and small ions.

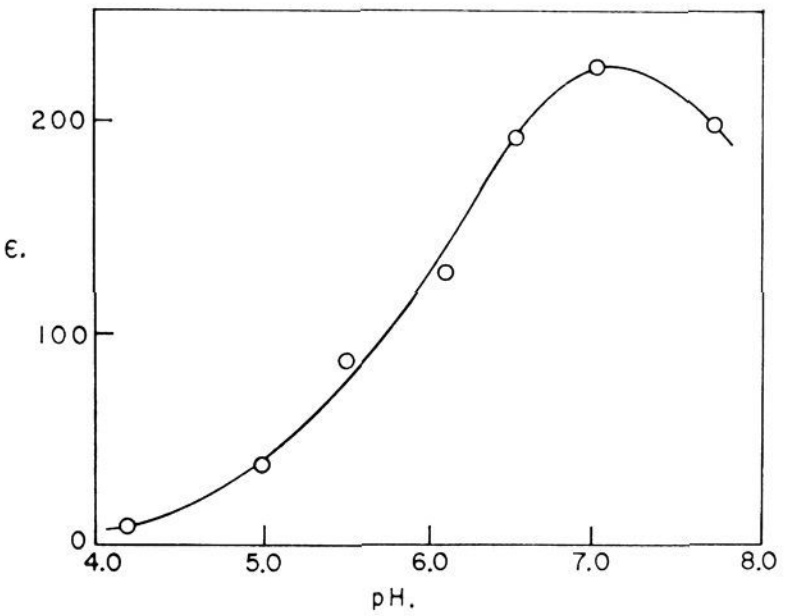


Fig. 4.—Effect of *p*H on initial height of 375 mμ band.

On this basis, it seemed reasonable to expect that the 375 mμ band should be abolished by the addition of other molecules with a known strong affinity for copper. Such indeed is the case for a variety of mercaptans, listed in Table I. In each case the concentration of R–SH added was equimolar with the protein, and the 375 mμ band disappeared immediately after addition of the mercaptan. Thus of the approximately ten copper ions present per mole of serum albumin, only one, that bound to the sulfhydryl group, reacts with the added mercaptan. By analogy with equation 3, therefore, we may write

$$\text{P—S—Cu} + \text{R—S}^- \longrightarrow \text{P—S—Cu—S—R} \quad (4)$$

Closer examination of details reveals, however, that the behaviors of OH^- and RS^- are not completely parallel. At *p*H 6, the RS^- concentration in a solution of 3×10^{-4} *M* total mercaptan is around 10^{-8} *M*[14], *i.e.*, of the same order as the OH^-

(12) It is recognized that actually the OH^- may be displacing an H_2O molecule from the coördination sphere of the copper ion, or perhaps an acetate ion present from the buffer.

(13) I. M. Klotz and W.-C. Loh Ming, THIS JOURNAL, **76**, 805 (1954).

(14) The *pK* of the mercaptans is assumed to be in the neighborhood of 10.

TABLE I

EFFECT OF VARIOUS REAGENTS ON 375 mμ ABSORPTION BAND[a]

Substance added	Concn. moles/l.	Optical density with reagent / Optical density without reagent
None		(1.00)
Salyrganic acid	0.0003	0.10
Hydrogen peroxide	.00015	.49
Thioglycerol	.00023	.22
Thioglycerol	.00011	.37
Thioglycerol	.00005	.65
Glutathione	.0003	.21
Mercaptosuccinic acid	.0003	.39
Cysteine	.0003	.22
Thiolhistidine	.0003	.41
Thiourea	.0003	.22
Cystine	.0003	.8
Methionine	.0003	1.0
Imidazole	.0003	1.0
Imidazole	.004	1.0
Potassium ferrocyanide	.0003	1.0
α,α-Dipyridyl	.0003	1.0
Potassium thiocyanate	.0003	1.0
Phosphate	.0003	1.0
Potassium cyanide	.0003	0.30
Sodium sulfite	.0003	0.27
Potassium iodide	.0003	0.9[c]
Sodium borohydride	.0003[b]	0.25

[a] All measurements were carried out in 0.2 *M* acetate buffer, *p*H 5.6–6.1, with (Cu^{++}) = 0.0033 *M* and (bovine albumin) = 0.0003 *M*. The ratios in the third column are taken from readings within the first five minutes. Readings were generally taken for periods up to 24 hours. [b] An increase of sodium borohydride to 0.0012 *M* abolished not only the 375 mμ peak but also practically removed the copper–albumin absorption maximum near 700 mμ. Presumably the borohydride reduced almost all the cupric ion. [c] The effect with iodide becomes much more marked if times longer than five minutes are used in this comparison.

concentration. The rate of disappearance of the 375 mμ peak is, however, too fast to be measured with the mercaptan, yet the change is extended over days with hydroxyl ion. *A priori* one might expect the OH^- with its very high mobility in aqueous solution to reach the copper more readily. The more rapid reaction with mercaptan may be due to the higher affinity of copper for sulfur than for oxygen. Nevertheless, the difference in behavior prompted us to carry out a different type of experiment.

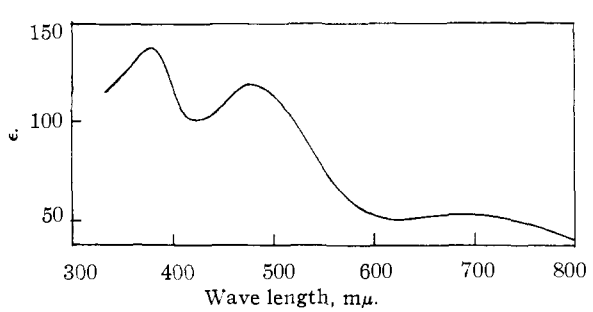


Fig. 5.—Spectrum of copper–albumin in presence of ferrocyanide, in 0.2 *M* acetate buffer, *p*H 6: (Cu^{++}) = 0.0033 *M*; (bovine albumin) = 0.0003 *M*; $(Fe(CN)_6^{-4})$ = 0.0003 *M*. In absence of ferrocyanide, peaks appear only at 375 and 700 mμ.

On the basis of equation 3 one might expect to remove the OH^- bound to Cu^{++} by bringing the protein complex into acid solution. If such an acid solution is then returned to *p*H 6, the 375 mμ band should be regenerated at first and then should slowly disappear once more as reaction (3) proceeds again. In practice we found that copper bovine albumin, which had been allowed to stand for four days until the 375 mμ peak had disappeared and reaction (3) had presumably gone essentially to completion, could be acidified to *p*H 2 with no untoward effects. However, as the *p*H of this solution was raised, protein precipitation ensued as soon as the *p*H exceeded 3. In contrast a control protein solution, with no copper added initially, could be carried through this same series of steps and the *p*H raised to 6 without difficulty. If cupric ion was added to the latter solution, the usual 375 mμ peak appeared. Clearly then, in the presence of copper, the protein suffered some irreversible alteration. Since this irreversible alteration is paralleled by the drop in 375 mμ absorption, it becomes difficult to ascribe the spectroscopic change to a reaction such as (3) which one would expect to be readily reversible.

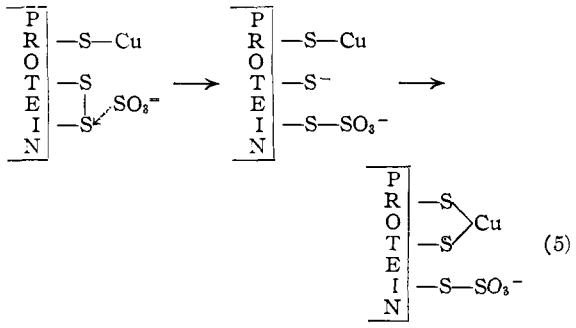
The effects of a variety of other copper-complexing agents were studied, therefore. The many compounds which were examined (Table I), other than mercaptans, fall into two classes insofar as their effect on the 375 mμ band is concerned. The amine-type compounds, such as imidazole and dipyridyl, are typical of those which produced no significant change in the rate of disappearance of the peak. On the other hand sulfite, cyanide and iodide changed markedly the kinetics of the optical changes. In all cases the concentration of added reagent was equimolar with the protein.

The failure of substances such as imidazole to produce a change in the 375 mμ band might be attributed to their relatively smaller affinity for copper and consequent failure to be bound. However, it can be shown, most readily with ferrocyanide, that the added reagent is bound to one of the cupric ions on albumin, but evidently not to that one linked to the mercapto group. Since the copper ferrocyanide complex has a peak in a very different region from those of copper albumin, it is evident (Fig. 5) that the ferrocyanide is bound to metal; in contrast to sulfite, cyanide and iodide, however, the rate of disappearance of the 375 mμ peak is unaffected by the ferrocyanide.

It seems doubtful, therefore, that the disappearance of the 375 mμ peak in the presence of sulfite, cyanide or iodide is due to a reaction analogous to equation 4. Serious question is also raised whether equation 3 is a correct description of the effect of hydroxyl ion.

The distinctive behavior of sulfite, cyanide and iodide parallels one other characteristic in which these three reagents (as well as the mercapto compounds) may be distinguished from the other copper complexers. Each of these three compounds is capable of splitting disulfide groups. One is attracted, therefore, to the possibility that the

special effects of these compounds on the 375 mμ peak comes from the closeness of an S–S side chain to the mercapto group of bovine albumin. With sulfite, for example, the reaction leading to the disappearance of the 375 mμ band may be visualized as



(5)

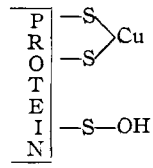
In the presence of copper, the neighboring S–S group, in contrast to the other disulfide groups in the protein, becomes most sensitive to sulfite. The reduction of this particular S–S group is facilitated by the fact that one of the products of the reaction, the $-S^-$ group, can be removed immediately by the adjacent copper; in fact the metal would facilitate formation of the activated complex.

Analogous reactions may be pictured with cyanide and iodide. The rates of cleavage in all cases are presumably speeded up in this protein system by the juxtaposition of the copper ion. In contrast to sulfite, cyanide, iodide and mercaptans, the other copper complexers are unable to bring about disulfide splitting. Consequently the vicinal position of –SH and S–S is of no advantage to these latter agents.

An equation analogous to (5) with OH^- substituted for $SO_3^=$ would also account more satisfactorily than equation 3 for the normal rate of disappearance of the 375 mμ peak. It is known that disulfide bonds are cleaved in strongly alkaline solution[15] though not normally in neutral solution. Under the present circumstances, however, the reaction may become appreciable even at *p*H 6 because of the catalytic effect of the neighboring copper ion. This situation thus becomes analogous to the catalysis by silver ions of the hydrolysis of cystine and of oxidized glutathione, described by Cecil,[16] which occurs even at a *p*H as low as 4.5. These observations have prompted us, furthermore, to examine the effect of cupric ion on certain non-protein disulfide linkages, normally stable in neutral aqueous solutions. The presence of metal ion induces spectroscopic changes, to be described in a separate publication, which show clearly the splitting of S–S linkages in a period of a few hours.

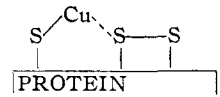
It is further of interest in connection with equation 5 that the addition of 1 mole of sodium borohydride, $NaBH_4$, per mole of protein also abolished the 375 mμ peak (Table I). Of the ten copper ions present it is the mercaptide-bound one that is affected. In view of the known ability of $NaBH_4$ to reduce disulfide linkages,[17] it seems likely that the effect with copper–albumin is analogous to that described by equation 5. Once again it appears that the juxtaposition of the mercaptide copper confers an unusual sensitivity on the adjacent disulfide linkage.

The use of an equation analogous to (5) to describe the mechanism of the normal disappearance of the 375 mμ band also provides a reasonable explanation of the denaturing effect observed after the removal of Cu^{++} in acid solution. When copper is removed from the structure



the protein remaining is very different from the original material, particularly in that a disulfide bond has been split and a second sulfhydryl group produced. Two sulfhydryl groups near the surface of the protein molecule could be very effective in producing aggregation by cross linking.

Unique Topology of Bovine Albumin.—The mechanism proposed to explain the disappearance of the 375 mμ absorption peak characteristic of the mercaptide-bound copper of bovine albumin also provides an explanation of the uniqueness of this protein in this phenomenon. Several other proteins (*e.g.*, ovalbumin, β-lactoglobulin, human serum albumin) known to have sulfhydryl groups have been examined but their copper spectra show no evidence of a peak at 375 mμ.[18] Human serum albumin is of particular interest since its content[3] of sulfhydryl is essentially identical with that of bovine serum albumin,[19] approximately 0.7 group per molecule. Even crystallized samples do not interact with cupric ion to give the characteristic 375 mμ band. With the picture underlying equation 5 one may visualize bovine albumin as containing a disulfide side chain in the neighborhood of the copper mercaptide:



This particular arrangement of side-chains is presumably not present in the other proteins.

The juxtaposition of a reducing group, $-S^-$, and an oxidizing group, –S—S–, bridged by a cupric ion, provides a mobile path for electrons. On this basis the exceptionally high extinction coefficient of the 375 mμ peak is understandable. The value of ε (140) shown in Fig. 1 has been computed on the basis of the total copper added. If one keeps in mind that only 0.7 mole of –SH is available per protein molecule and that this figure sets the upper limit for mercaptide-bound copper, the value of ε becomes nearly 2000. For the violet end of the visible spectrum, molecular extinction coefficients of copper complexes rarely exceed 100.

(15) See for example E. Barbu, J. Lessiau and M. Macheboeuf, *Bull. soc. chim. biol.*, **31**, 1254 (1949).

(16) R. Cecil, *Biochem. J.*, **47**, 572 (1950).

(17) E. V. Jensen, private communication.

(18) No evidence of a peak was obtained with pepsin or bovine fibrinogen either. Attempts to examine α- and β-casein failed because their copper complexes were insoluble at the concentrations necessary to detect the 375 mμ band.

(19) R. Benesch and R. E. Benesch, *Arch. Biochem.*, **19**, 35 (1948).

On the basis of the preceding interpretations, it is possible to estimate the distance between the sulfhydryl and disulfide side-chains in bovine albumin. Taking the covalent radius[20] of copper as 1.35 Å. and assuming that the metal would retain a square-planar coördination, the sulfide to disulfide distance becomes approximately 3.4 Å.

Effect of Copper Concentration; Configurational Changes in Protein.—At *p*H 6 in acetate buffer, the appearance of the 375 mμ peak with bovine albumin is markedly sensitive to the mole ratio of copper:protein. Although bovine albumin contains slightly less than one mercaptan group per mole, the addition of one mole of cupric ion gives no indication of an absorption peak at 375 mμ.[21] It is only when approximately four moles of copper ion have been added that the suggestion of a shoulder at this wave length appears.[22] Thereafter the peak rises very rapidly with further increase in metal concentration (Fig. 6).

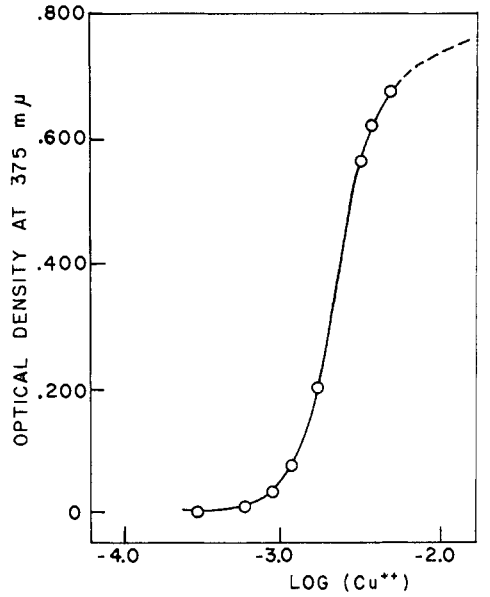


Fig. 6.—Effect of copper concentration on initial height of 375 mμ band.

The steepness of ascent in Fig. 6 is much too great to be accounted for by any simple mass-law treatment. It seems likely, therefore, that some coöperative phenomenon is involved. From published work on the binding of copper by bovine albumin[23] it is evident that most of the metal ion is on the protein at total metal ion concentrations in the neighborhood of 10^{-3} *M* in acetate buffer. It seems, therefore, that in the present experiments the uptake of Cu^{++} in the range of 2 to 10 moles per mole of protein produces some configurational change in the albumin molecules so that the mercaptan and disulfide groups, which are required for the 375 mμ peak, are placed in suitable juxtaposition.

Some attempts were made to disturb the configuration favorable for the 375 mμ peak by conventional denaturing methods. In the presence of 6 or 8 *M* urea, the peak disappears (Fig. 7). However, this effect cannot be ascribed unequivocally to a configurational disturbance, for the urea[24] may be competitively complexing with the copper. Exposure of bovine albumin to 8 *M* urea (for one-half hour) followed by dialysis to remove the urea gives a protein which on addition of copper shows the absorption peak at 375 mμ (Fig. 7). This return seems to be essentially complete. Thus, at least in the presence of copper, the protein configuration in the neighborhood of the sulfide-disulfide side chains seems to remain remarkably stable.

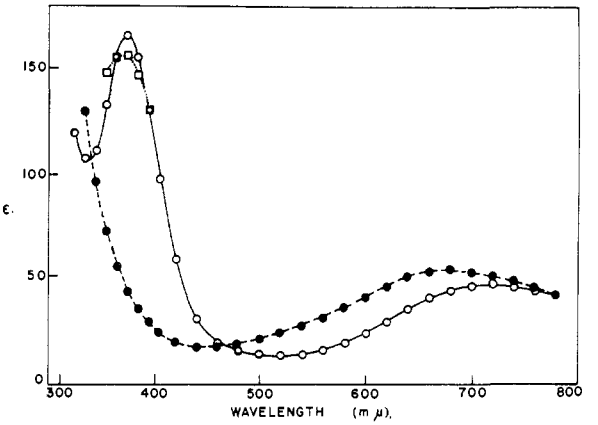


Fig. 7.—Effect of urea on spectrum of copper-bovine albumin. All spectra taken in 0.2 *M* acetate buffer *p*H 6.2, with (Cu^{++}) = 0.0033 *M* and (protein) = 0.0003 *M*: O, bovine albumin, in buffer alone; ●, bovine albumin in buffer containing 8 *M* urea; □, bovine albumin exposed to 8 *M* urea and dialyzed to remove urea.

Conclusion.—Of the 10–20 copper ions which can be put into bovine albumin (without precipitation of protein), only one can be placed in the special sulfhydryl–disulfide locus. Only this one reveals, by a change in optical properties, the chemical changes wrought by the presence of the metal. It seems likely, however, that the catalytic effect of copper on disulfide splitting is not limited to metal bound to a neighboring mercapto group. In experiments with disulfides in relatively simple molecules, *e.g.*, dithiodisuccinic acid, we have found that the presence of cupric ions, bound presumably to carboxyl groups adjacent to the S–S, catalyzes markedly the hydrolysis of the disulfide linkage well below *p*H 7. The studies of Cecil[16] indicate that other metals may have similar properties. Thus it becomes clear that orientations of neighboring groups in a protein molecule play a dominant role in providing conditions favorable

(20) L. Pauling, "The Nature of the Chemical Bond," Cornell Univ. Press, Ithaca, N. Y., 1939, pp. 154, 167. The radius given for copper is actually for a tetrahedral bond.

(21) Likewise even if 10 moles of cupric ion are present per mole of albumin, but 0.015 *M* citrate is present in place of acetate as buffer, no 375 mμ peak is observed. In citrate buffer the binding of copper by this protein is about 0.1 that in acetate.[18]

(22) At a cupric ion:protein ratio of 2, spectra were examined over a range of protein concentration from 2 to 10%. In no case was a peak observed at 375 mμ.

(23) H. A. Fiess and I. M. Klotz, THIS JOURNAL, **74**, 887 (1952).

(24) Attempts to replace urea by guanidine hydrochloride failed because the added cupric ion caused precipitation. Exposure to temperatures of 60–70° for 30 minutes did not affect the appearance of the 375 mμ peak upon addition of copper. Higher temperatures led to coagula whose spectra could not be examined.

to the expression of the catalytic properties of metals.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, WEIZMANN INSTITUTE OF SCIENCE]

Theoretical Analysis of the Polymerization Kinetics of N-Carboxy-α-amino Acid Anhydrides

BY EPHRAIM KATCHALSKI, YECHIEL SHALITIN AND MATATIAHU GEHATIA

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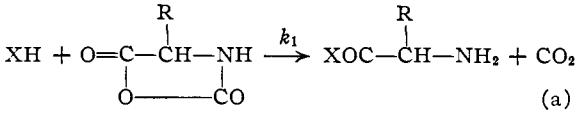
A theoretical analysis is given for the kinetics of polymerization of N-carboxy-α-amino acid anhydrides, assuming an initiation reaction (a), with a specific rate constant k_1, a propagation reaction (b), with a specific rate constant k_2, and termination reactions (c) and (d), with the respective specific rate constants k_3 and k_4. Formulas were derived for the rate of polymerization and for the rate of carbon dioxide evolution as well as for the molecular weight distribution and average degrees of polymerization of the still growing chains, characterized by free terminal amino groups, and of the terminated peptide chains, characterized by terminal carboxyl groups. From the general treatment three special cases were derived. Case I, $k_1 = k_2$; $k_3 = k_4 = 0$. The formulas given for this case were shown to agree with those derived by Flory[3] for the polymerization of ethylene oxide as well as with those of Ballard and Bamford[5] deduced for the polymerization of N-carboxy-DL-phenylalanine anhydride and N-carboxy-DL-leucine anhydride initiated by preformed polymer. Case II, $k_1 \neq k_2$; $k_3 = k_4 = 0$. The formulas for this case were found identical with those of Breitenbach and Allinger[6] derived for the polymerization of N-carboxy-DL-phenylalanine anhydride initiated by amines of different basicity. Case III, $k_1 = k_2$; $k_3 = k_4$. The variation with time of the molecular weight distribution of the growing and terminated chains was calculated assuming particular values for k_2 and k_3. It was shown that while the molecular weights of the terminated chains are spread over a wide range, the molecular weights of the unterminated chains are concentrated in the high molecular weight fraction of the polymer. Possible methods for the evaluation of k_1, k_2, k_3 and k_4 are suggested and the conditions necessary for the preparation of poly-α-amino acids of high molecular weight are discussed.

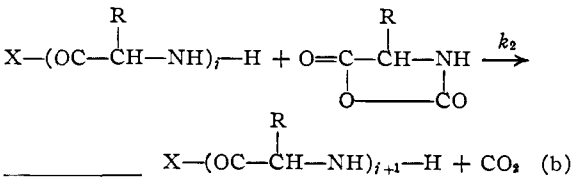
In a previous article[1] it has been shown by Sela and Berger that the polymerization of N-carboxy-α-amino acid anhydrides initiated by amines or alcohols (XH, X = R_1R_2N or RO) involves not only initiation and propagation reactions (a) and (b), but also a termination reaction (c) (see below). In the initiation reaction the X-residue of the initiator attaches itself to the C-5 carbonyl of the oxazolidine-2,5-dione, carbon dioxide is evolved, and a free amino group appears. During propagation new peptide bonds are formed as a result of the reaction of the free amino groups of the growing chains with the C-5 carbonyl groups of the anhydride. There is, however, no change in the total number of the amino groups. The termination reaction (c) causes the disappearance of the free amino groups of the growing chains as a result of their interaction with the C-2 carbonyl groups and the formation of peptides with terminal carboxyl groups adjacent to an urea link. It is obvious that the catalyst may be eliminated from the system by analogous termination reaction (d).

Initiation:



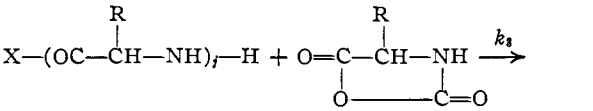
(a)

Propagation:

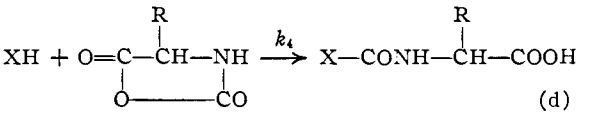


(b)

Termination:



(c)



(d)

In the following a general kinetic analysis of a polymerization, proceeding according to the scheme described above, will be given. From the general treatment some limiting cases of special interest will be derived.

General Case.—Here it will be assumed that the polymerization is determined by four different constants k_1, k_2, k_3 and k_4, of the reactions (a), (b), (c) and (d), respectively. The concentration of the N-carboxy-α-amino acid anhydride is denoted by (M), the concentration of the catalyst by (XH), the concentration of the "growing monomer," consisting of one amino acid residue and containing a free terminal amino group by N_1^*, and the concentration of a growing peptide composed of j amino acid residues and containing a free terminal amino group by N_j^*. The rate of disappearance of catalyst is given by eq. 1, the rate of formation of a growing monomer by eq. 2, and the rate of formation of a growing j-mer by eq. 3.

$$-\frac{d(XH)}{dt} = (k_1 + k_4)(M)(XH) \qquad (1)$$

$$\frac{dN_1^*}{dt} = k_1(M)(XH) - (k_2 + k_3)(M)N_1^* \qquad (2)$$

(1) M. Sela and A. Berger, THIS JOURNAL, 77, 1893 (1955). *Cf.* also M. Sela and A. Berger, *ibid.*, 76, 6350 (1953).