II. 1. SOME CHEMICAL PROPERTIES OF THE SULFHYDRYL GROUP IN BOVINE PLASMA ALBUMIN

Elwood V. Jensen

The University of Chicago, Chicago, Illinois

I. Introduction

The sulfhydryl group of plasma albumin is an entity which, to my mind anyway, possesses a number of extremely interesting and somewhat puzzling properties. Although earlier work indicated the presence of some sulfhydryl in this protein, the first substantial evidence that there actually is a sulfhydryl group in albumin was obtained by Hughes (*1*) who treated human plasma albumin, isolated and crystallized by the Harvard procedure, with mercuric chloride and observed that two-thirds of the protein was precipitated as a dimeric molecule, presumably linked together by mercury through sulfhydryl groups. Shortly thereafter Benesch and Benesch (*2*), using a modification of Kolthoff's amperometric titration procedure for mercaptans, found that crystalline bovine plasma albumin reacted with about three-fourths of an equivalent of ammoniacal silver nitrate per mole of protein. These observations suggested that human and bovine plasma albumin may consist of two types of material: about 70% is a protein containing one sulfhydryl group per albumin molecule which Hughes has termed "mercaptalbumin"; 30% either has no sulfhydryl group or else has a sulfhydryl of considerably different reactivity. The latter conclusion is supported by more recent observations of Benesch and associates (*3*) that if the amperometric titration with silver nitrate in tris(hydroxymethyl)aminomethane buffer is carried out in the presence of urea, one full equivalent of sulfhydryl per mole of albumin is titrated.

Our own interest in the sulfhydryl stems from observations of a profound effect of a single molar equivalent of certain "sulfhydryl reagents"

on the aggregation phenomena which accompany the denaturation of plasma albumin by heat (*4*) or urea (*5*). To explain how a single chemical group can influence the aggregation behavior of a large molecule containing nearly 600 amino acid residues, we postulated that, under conditions of protein denaturation, the sulfhydryl group can initiate a chain reaction with disulfide groups leading to the formation of many intermolecular disulfide bonds. Subsequent observations by several investigators, including Kauzmann (*6*), Halwer (*7*), Sanger (*8*), Edsall (*9*), and Kolthoff (*10*), have supported and extended this concept.

So we see that in plasma albumin there is a chemical entity, apparently a sulfhydryl group, which reacts with mercuric ions to the extent of about 70% and with silver ions in amperometric titration to the extent of either 70% or 100%, depending on the medium. Under ordinary conditions this group does not react strongly with nitroprusside but the aggregation studies show that it reacts practically quantitatively with iodoacetamide. Of course one can put his mind at ease about these phenomena by resorting to what yesterday was termed the "dogma of accessibility" and refer to the sulfhydryl group as "masked." And since the treatment with iodoacetamide is usually carried out overnight, one has only to assume that at midnight the traditional unmasking takes place so that in the morning one finds his sulfhydryl completely reacted with iodoacetamide. But to us the behavior of the albumin sulfhydryl seemed somewhat unconventional, and since in the minds of many, unconventional behavior is equated with subversive, and since subversive activities of course require investigation, being loyal citizens of the scientific community, we proceeded to investigate.

II. Interaction with Silver Ions

The first study that we undertook was the binding of silver cations to bovine plasma albumin as a function of the silver concentration (*11*). Here we expected that as the silver concentration is increased from zero, a point would be reached where silver would be bound to the sulfhydryl group and that this association should commence at a rather low silver concentration (Fig. 1). As the silver concentration is raised further and all the sulfhydryl groups have reacted, one should observe a plateau in the silver binding curve until the silver concentration reaches a point where begins nonspecific cation binding to groups such as carboxyl. The height of the plateau should indicate the number of sulfhydryl groups in the molecule, and from the mid-point of the initial slope one should be able to calculate the dissociation constant of the silver-sulfhydryl complex of bovine plasma albumin.

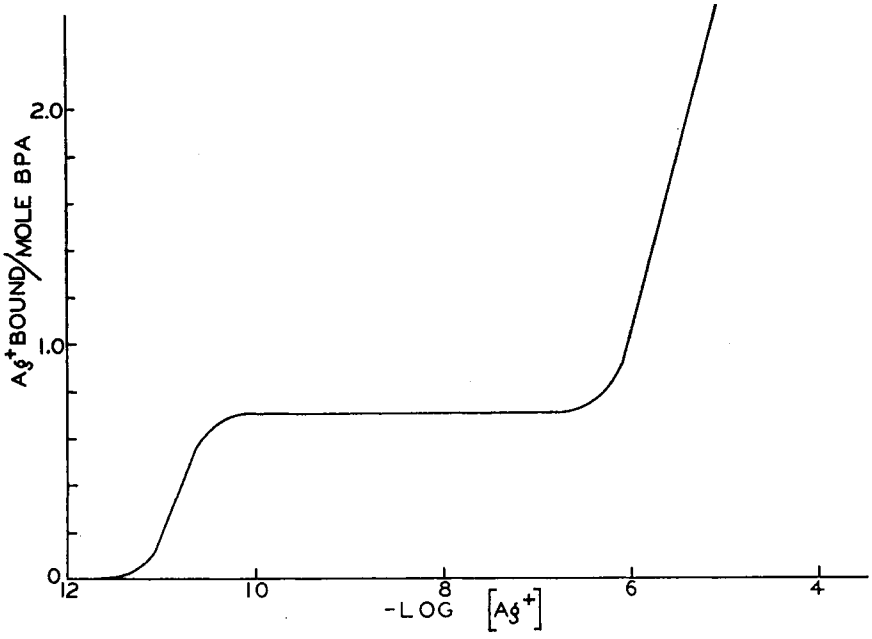

FIG. 1. Expected curve for the binding of silver ion to bovine plasma albumin as a function of silver concentration.

So, being naive and trusting, we set out to obtain this curve employing the familiar equilibrium dialysis technique utilized so effectively by Klotz and co-workers for the study of the binding of small molecules to proteins. For the determination of the silver concentration inside and outside the dialysis bag, we employed radioactive silver nitrate (Ag^{110}) of specific activity sufficient to permit counting of silver concentrations down to 10^{-12} M. With these dilute solutions we encountered certain technical difficulties which are rather interesting. In the first place silver ions have a great affinity for glass so that if one wants to have any silver remaining in a dilute silver nitrate solution it is necessary to use polyethylene vessels. Similarly one cannot deliver an aliquot for counting from a glass pipette since most of the activity remains on the pipette wall. Fortunately Ag^{110} is a γ-emitter so that one can count aliquots conveniently by using a rather short pipette and counting the pipette together with the aliquot in a well-type counter. Between experiments the pipettes are cleaned with a dilute solution of hydrofluoric acid in nitric acid (*12*) to remove the silver ions bound to the glass. Finally, dialysis tubing also has a great capacity for binding silver ions so that it is necessary to first treat this tubing with methyl iodide which eliminates 90% of its capacity for binding silver.

With these technical difficulties eliminated, we then proceeded to study the binding of silver ions to bovine plasma albumin and found to our surprise that we did not obtain a curve resembling Fig. 1 at all. Rather we found that at silver ion concentrations below 10^{-7} M there is practically no binding of silver to albumin (Fig. 2). As the silver concentration is increased toward 10^{-6} M, argentation begins to take place. However, there is no indication of any plateau at either 0.7 or 1.0 mole of silver

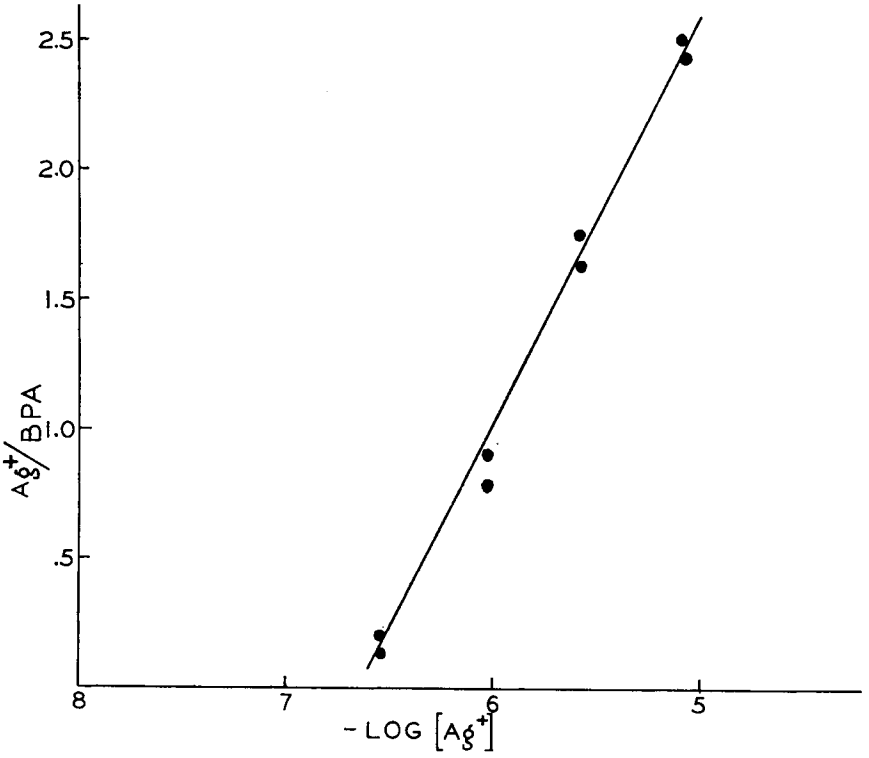

FIG. 2. Observed binding of silver ion to bovine plasma albumin. Protein 1×10^{-5} M in 0.05 M citrate buffer, pH 6.1.

per mole of protein, but rather the number of silver ions bound continues to increase with the logarithm of the silver concentration in a manner suggestive of nonspecific cation binding. Association of 10^{-5} M albumin with silver first commences at about 2×10^{-7} M silver ion concentration no matter whether the experiment is carried out in borate buffer at pH 10 or in citrate buffer at pH 6 or 3, although the higher the pH the more rapidly the binding increases at higher silver concentrations (Fig. 3).

In contrast to the situation in borate buffer at pH 10, if the equilibrium

dialysis is carried out in ammonia-ammonium nitrate buffer of pH 10, 0.76 moles of silver was observed bound to albumin, in good agreement with the amperometric titration of this albumin sample in the same medium. Under these conditions, the concentration of free silver ion is only 10^{-11} M, although it must be remembered that the total silver present as

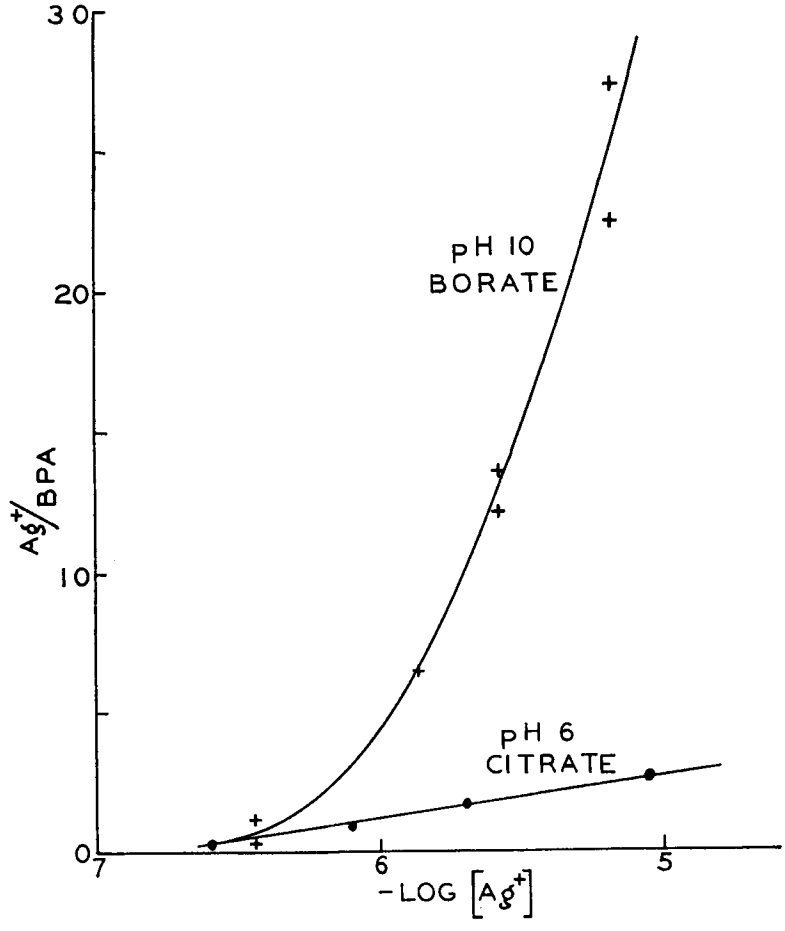

FIG. 3. Effect of pH on the binding of silver ions to bovine plasma albumin.

the ammonia complex is 10^{-5} M. If it is the free silver ion which binds to sulfhydryl, it follows that ammonia must exert a marked effect on the reactivity of the protein sulfhydryl toward silver ions. In contrast to ammonia, the presence of 6 M urea at pH 6 did not promote binding at lower silver concentrations and actually decreased the binding somewhat at higher silver concentrations.

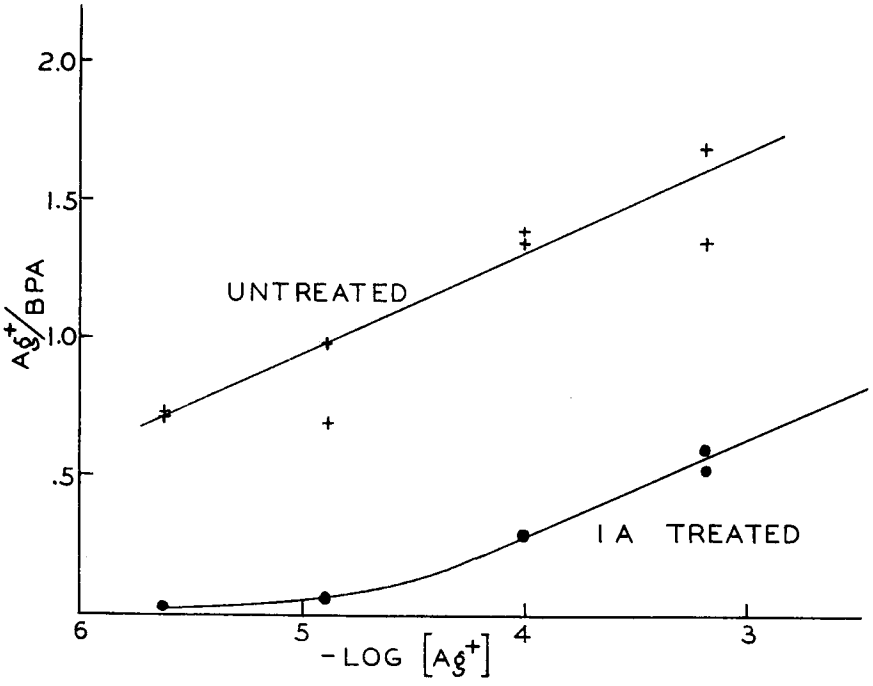

FIG. 4. The effect of iodoacetamide treatment on the binding of silver ions to bovine plasma albumin in pH 3.0 citrate buffer (6 moles of iodoacetamide per mole of albumin).

Since the silver-binding curves obtained appear to be of nonspecific nature reaching rather large numbers of bound silver ions, one might wonder where what we call the sulfhydryl group fits into the picture. Interestingly enough one binding site of albumin is clearly different from the rest in that it can be completely eliminated by previous treatment of the protein with iodoacetamide. Figure 4 shows the decrease in argentation at pH 3 following exposure of the albumin to six equivalents of iodoacetamide while Fig. 5 shows the effect of varying amounts of iodoacetamide on the silver binding at pH 6 with two different silver ion concentrations. It is clear that just one mole of silver-binding capacity can be eliminated from each mole of albumin, although it takes a little more than two moles of iodoacetamide to do the job completely.

III. Nature of Sulfhydryl in Albumin

From these observations, coupled with the previously mentioned findings of others, I believe we can draw some conclusions relevant to the true identity of the masked marvel known as albumin sulfhydryl.

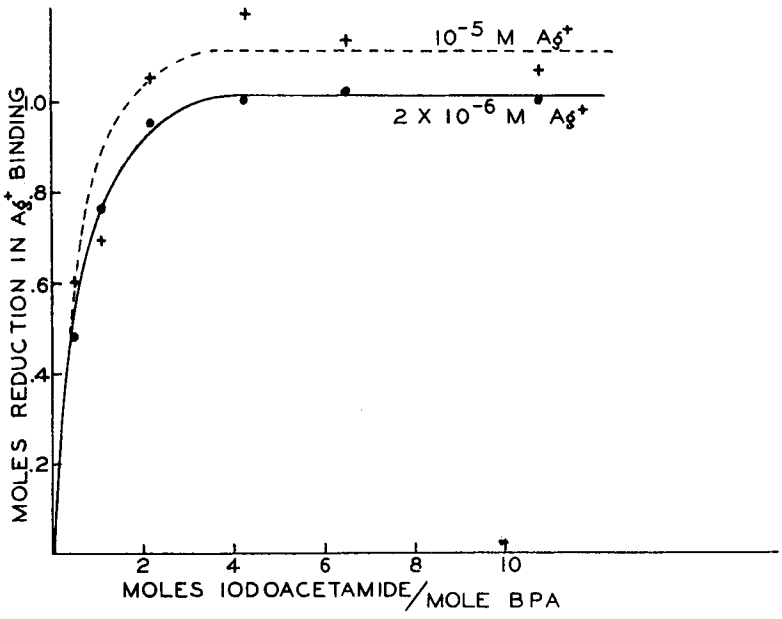

FIG. 5. The reduction in silver binding by treatment of bovine plasma albumin with varying amounts of iodoacetamide. Binding in citrate buffer, pH 6.1.

First, the total amount of the entity which reacts with iodoacetamide and, under certain conditions, with silver ions is one group per protein molecule rather than some fraction of one. Second, the affinity of this group for silver ions is much less than one would expect for a sulfhydryl group, being of the same order of magnitude as the nonspecific cation binding. Third, in the presence of ammonia the interaction of this group, or at least of some group in the protein molecule, takes place at extremely low free silver ion concentrations.

As an explanation for this syndrome, I would like to submit for your consideration the proposal that the sulfhydryl group of bovine plasma albumin does not exist as such but rather in extremely stable but reversible combination with some other group in the protein molecule* (Fig.

* Note added in proof: It remains to be determined whether the sulfhydryl group participates in a thiazoline structure, which has been proposed by K. Linderstrøm-Lang and C. F. Jacobsen (*J. Biol. Chem.* **137**, 443 (1941) and shown by M. Calvin (*in* "Glutathione" [S. Colowick *et al.*, eds.), p. 3. Academic Press, New York, 1954] to be formed under certain conditions with glutathione. The disruptive effect of ammonia on the albumin sulfhydryl linkage would be compatible with such an explanation. The present observations merely provide evidence that an intramolecular "sulfhydryl bond" of some kind exists in the native bovine albumin structure. That sulfhydryl groups may be able to hold protein units together in a reversible manner has been suggested by N. B. Madsen and C. F. Cori (*J. Biol. Chem.* **223**, 1055, 1956) on the basis of the reversible disaggregation of phosphorylase by *p*-chloromericuribenzoate.

6). Thus, even though the equilibrium for reaction II lies far in the direction of argentation, the intramolecular association of the sulfhydryl group is sufficiently strong to render the over-all affinity constant for silver comparatively low. Thus an unexpectedly high silver concentration is required before argentation of the sulfhydryl group takes place to any appreciable extent. Iodoacetamide, on the other hand, reacts irreversibly with sulfhydryl and thus can tie up all the sulfhydryl group in stable thioether linkages. The presence of ammonia must in some way weaken the intramolecular sulfhydryl association shifting the equilibrium in the direction

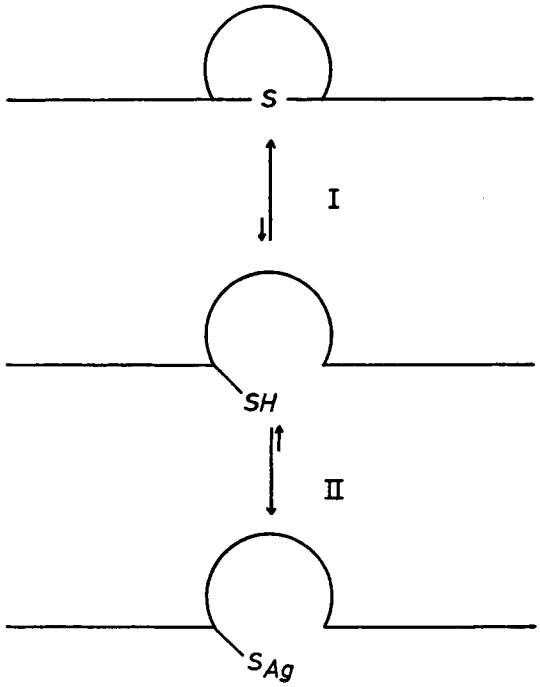

FIG. 6. Proposed equilibrium phenomena in bovine plasma albumin.

of argentation at lower silver concentrations. Whether the reaction of mercury and, under some conditions, of silver to the extent of only 0.7 moles per mole of albumin really means that two-thirds of the albumin is different from the other third or whether under the conditions employed the equilibrium of reaction II just happens to permit two-thirds of the sulfhydryl groups to react with the heavy metal is open to question, although it would seem more than coincidental that the figure ⅔ should be observed both with mercury and with ammoniacal silver ions.

The foregoing concept of stable intramolecular association of the

albumin sulfhydryl group also provides an explanation for what to us was a puzzling phenomenon repeatedly observed in previous studies of the influence of the sulfhydryl group on the denaturation of albumin by urea (5). This is the fact that immediately following exposure to urea the viscosity of a solution of sulfhydryl-blocked albumin is always somewhat higher than that of a similar solution of sulfhydryl-containing albumin, although the viscosity of the latter soon increases to a higher value because of the sulfhydryl-initiated cross-linking chain reaction with disul-

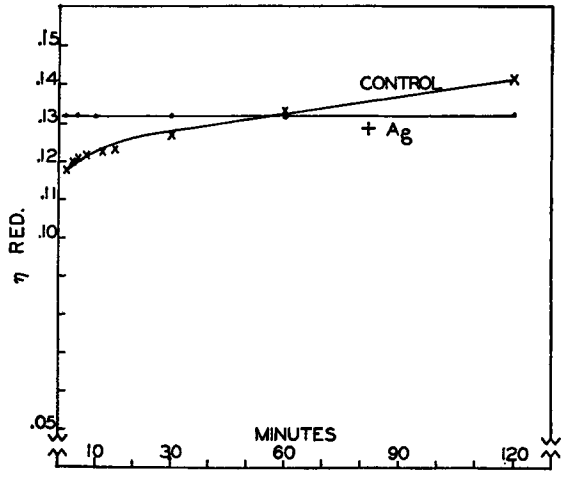

FIG. 7. Viscosity of solutions of 1% bovine plasma albumin following exposure to 6 *M* urea, KEY: x, sulfhydryl-containing albumin; ●, sulfhydryl-blocked albumin. (0.1 *M* phosphate buffer; pH 8.0, at 30°.)

fide groups (Fig. 7). On the basis of Fig. 6, it would appear reasonable that sulfhydryl-blocked albumin with the intramolecular sulfhydryl linkage broken can unfold quickly in urea to a greater extent than can albumin with the sulfhydryl linkage intact, leading to higher initial viscosity reading in the case of the sulfhydryl-blocked albumin. It should be pointed out, however, that before treatment with urea there is no detectable difference in the viscosities of solutions of sulfhydryl-blocked and sulfhydryl-containing albumin.

IV. Interaction with Cupric Ions

In conclusion I would like to utilize the foregoing concept of protein sulfhydryl structure to explain one additional rather mysterious and unconventional phenomenon of protein chemistry, namely, the ultraviolet absorption peak at 375 mμ reported by Klotz and co-workers (*13*) to re-

sult from the interaction of cupric ions with bovine plasma albumin. We, too, have carried out a rather extensive spectrophotometric study (*14*) of the interaction of copper and albumin and have repeated and confirmed many of the observations of Klotz, although on the basis of certain additional experiments we differ with him as to the interpretation of these facts. Unfortunately, time does not permit a complete presentation of his or our investigations, but I will briefly refresh your memory about the main features of the phenomenon and then point out the relation to the proposal of Fig. 6.

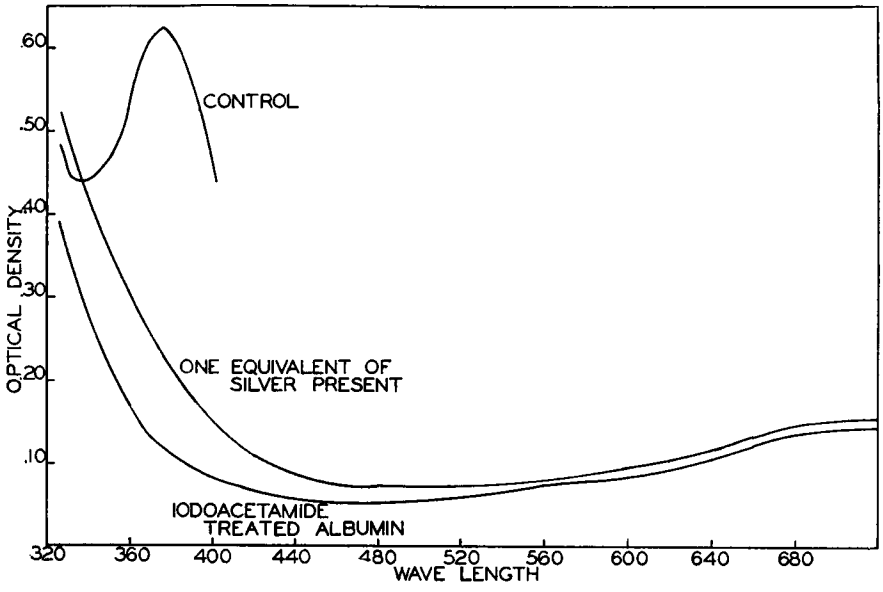

FIG. 8. Ultraviolet absorption spectra of the cupric complex of bovine plasma albumin. (Albumin 17 mg./ml.; pH 6.1; Cu/albumin ratio = 11.8.)

When a solution of bovine plasma albumin is treated with copper nitrate, the first mole of copper added gives rise to a rather weak but well defined absorption band at 530 mμ which in no way involves the sulfhydryl group. A similar 1 to 1 copper-albumin complex independent of sulfhydryl has been observed by Kolthoff by amperometric titration techniques (*15*). The second mole of copper has no specific effect in the absorption spectrum but with the third mole of copper an absorption band appears at 375 mμ (Fig. 8) which increases in intensity with increasing ratio of copper to albumin. This 375 mμ absorption is completely eliminated by blocking the albumin sulfhydryl group by either pretreatment with iodoacetamide or by the addition of one mole of silver ion. Because

the presence of copper increases the end absorption with both sulfhydryl-containing and sulfhydryl-free protein, one finds it advantageous to measure the intensity of the 375 mμ peak against a blank containing albumin, copper, and silver, which gives what in the accompanying figures is called the "corrected absorption," rather than against albumin solutions alone which gives what is called the "total absorption."

Figure 9 shows how the intensity of the 375 mμ peak increases with increasing copper to albumin ratio. The maximum intensity (either total or corrected) is observed at a ratio of from 10 to 14 copper ions per albu-

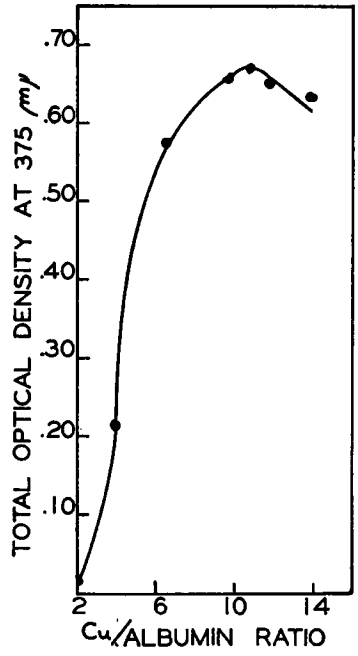

FIG. 9. The effect of the copper-albumin ratio on the 375 mμ absorption. Five minutes after mixing. (Albumin 17 mg./ml.; pH 7.2.)

min molecule, the exact optimum depending on the sample of albumin used. This dependence on the amount of copper is not a mass action effect, since the 375 mμ absorption was found to obey Beer's law over the protein concentration range $5 \times 10^{-6} M$ to $4 \times 10^{-4} M$ keeping the copper-albumin ratio at 11 (Fig. 10). Moreover, the same copper-albumin ratio was required for maximum absorption with $2.4 \times 10^{-5} M$ albumin as with $2.4 \times 10^{-4} M$.

The 375 mμ absorption peak forms rapidly as albumin and cupric nitrate are mixed and fades slowly with time (Fig. 11); the rate of fading

is increased by high pH and copper concentrations but is independent of the presence of oxygen. As the peak fades, the sulfhydryl content of the protein (as determined by amperometric titration with silver in the presence of Versene to tie up the copper) falls at a roughly comparably rate (Fig. 12).

These are the main experimental facts in our own investigation concerning the interaction of albumin with cupric ions, most of which confirm reported observations of Klotz. To me the most interesting and puzzling aspect is that a rather large number of cupric ions are required for

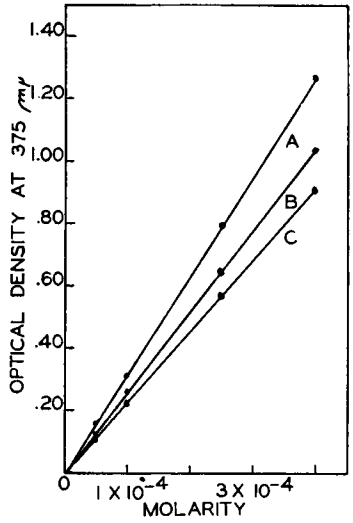

FIG. 10. Relation of the 375 mμ absorption to albumin concentration. A. Total optical density 5 minutes after mixing. B. Corrected optical density 5 minutes after mixing. C. Corrected optical density 27 minutes after mixing. (Cu/albumin ratio = 11.0; pH = 7.2.)

the maximum absorption and yet the whole phenomenon depends in an all-or-none fashion on the presence of a single sulfhydryl group. The Beer's law dependence indicates that the need for several cupric ions per albumin molecule is not a mass action effect. We are left with the question, just as we were several years ago in the aggregation studies, as to how a single group in the protein molecule can promote a reaction which would appear to involve a large number of reacting groups. In this case we were unable to come up with any kind of a chain reaction, but we would like to suggest that the sulfhydryl group exerts a "permissive effect" on the reaction of cupric ions with some chemical group at a number of sites in the albumin molecule. Utilizing the concept of Fig. 6, one can

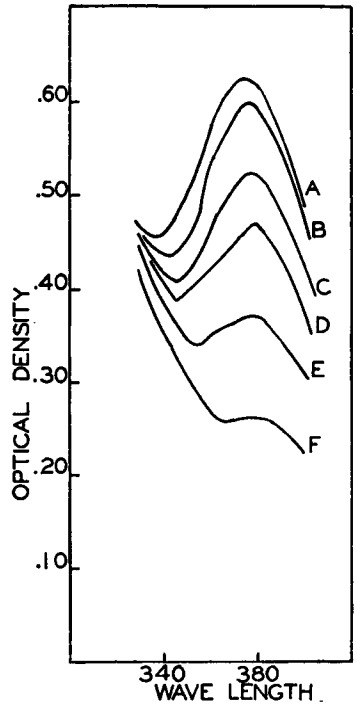

FIG. 11. Fading of the 375 mμ absorption. Time after mixing: A, 10 minutes; B, 25 minutes; C, 50 minutes; D, 80 minutes; E, 4 hours; F, 22 hours. (Albumin 17 mg./ml.; pH 6.1; Cu/albumin ratio = 11.8.)

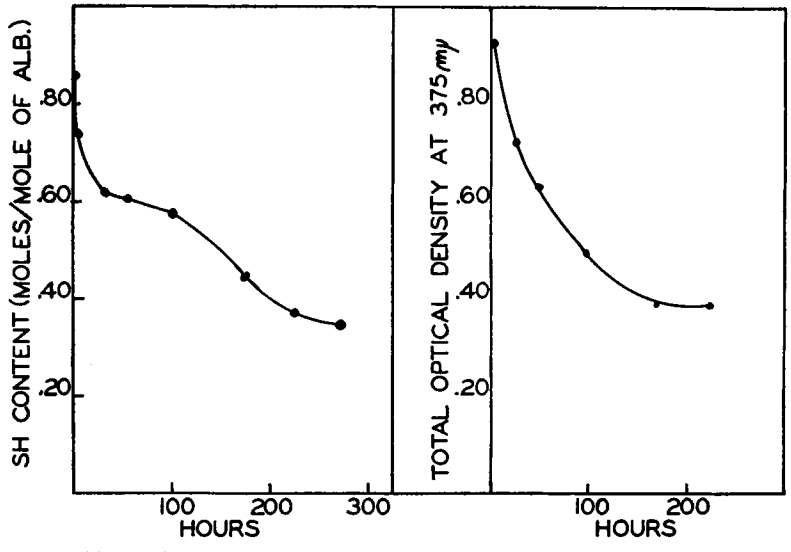

FIG. 12. Change in sulfhydryl content and 375 mμ absorption of copper-albumin on standing. (Albumin 17 mg./ml.; pH 7.3; Cu/albumin ratio = 11.)

consider that the intramolecular sulfhydryl linkage holds the protein in a specific configuration necessary for the formation of chromophoric complexes with copper, and that disruption of this configuration by reaction of the sulfhydryl group with silver or iodoacetamide prevents completely the formation of the chromophore. This is not very different from the situation in which blockage of a sulfhydryl group in the enzyme, glyceraldehyde phosphate dehydrogenase, prevents the binding of a pyridine nucleotide coenzyme at another site on the protein molecule.

V. Conclusion

In summary, we feel that the concept of stable but reversible intramolecular association of the albumin sulfhydryl serves to explain a number of well established but rather mysterious properties of this group: in particular (a), its relatively low affinity for silver ions but its ready reaction with iodoacetamide; (b), the fact that, after treatment with urea, the initial viscosity of a solution of sulfhydryl-blocked albumin is always higher than that of a similar solution of sulfhydryl-containing albumin; and (c), the ability of a single sulfhydryl group to promote chromophore formation between the albumin molecule and a rather large number of cupric ions.

Whether or not any or all of the aspects of this proposal are regarded with favor, I am sure it will be agreed that what we call the sulfhydryl group of serum albumin is a most interesting entity capable of exerting an influence on certain properties of the whole protein molecule far out of proportion to the small amount of this group present.

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Discussion

BENESCH: Dr. Klotz already pointed out in his original paper on the copper spectrum that in the sample of human serum albumin which he tried, the copper spectrum was absent. We have checked this with whole fresh serum and there is no doubt that while you can see the spectrum very clearly in bovine serum, it is absent in human serum (Fig. A). The two proteins therefore are entirely different as far

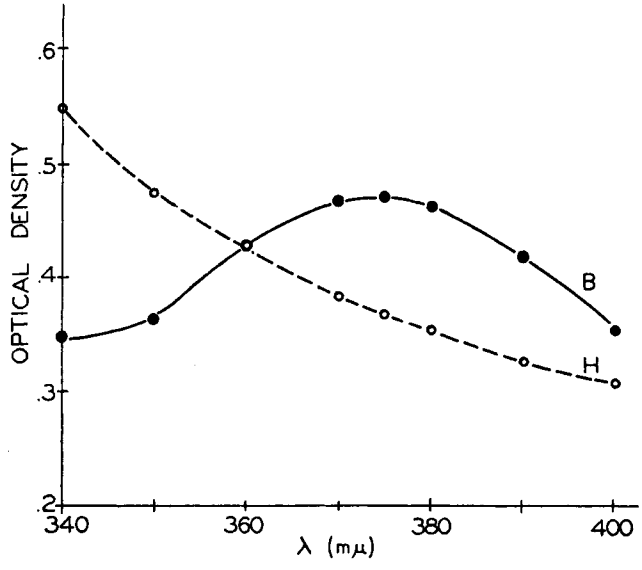

FIG. A. Copper spectra of whole serum. H = Human serum, B = Bovine serum; both contain 10 moles of copper per mole of albumin.

as the copper spectrum is concerned. Since, on the other hand, they behave entirely similarly with respect to the reactivity of the —SH group, I do not see how you can use the same model to account for the two phenomena.

One other short comment concerns the combination of silver with the sulfhydryl group of serum albumin. Some years ago (*Federation Proc.* **15**, 218, 1956), we observed that two-thirds of an —SH group combines with silver in water, but a whole one combines with silver in 8 *M* urea. On the other hand when in the very same solution the urea is diluted from 8 *M* to 1 *M*, one-third of the silver pops right out again,

and only two-thirds of an equivalent remains combined. This extra third of silver evidently has much less affinity and is, therefore displaced again as soon as the urea becomes sufficiently dilute.

HAUROWITZ: I would like to ask two questions. Since you get binding of one silver in ammonia only, would it not be possible that actually the diammine silver complex is bound? I wonder whether you get the same result if you titrate in cyanide. Maybe the silver amine complex is bound and not the silver ion? In horse serum albumin one of the cystine molecules has a free amino group. I do not know whether it could have anything to do with these phenomena.

JENSEN: In answer to your first question about titrating with silver cyanide, I do not know the result since we have not done this. It is possible that the complexed silver binds to the sulfhydryl, but somehow one has the feeling that silver already complexed with ammonia would not react with a sulfhydryl group unless it expelled the ammonia. Perhaps the inorganic chemists can speak with more authority on this point.

KOLTHOFF: I think it would be very hard to titrate with silver cyanide because it is more stable than the silver mercaptide. As far as the sulfhydryl is concerned, I do not think there is general agreement about the sulfhydryl content of albumin. I am not doubting the experiments of Dr. Benesch. I can only say that in our work we have been titrating amperometrically with silver nitrate at pH 9, but we also got 0.67 in 200 determinations at pH 7. Then we decided to change to mercury chloride as a reagent, and it does not make any difference whether we use native albumin or denature with urea or guanidine hydrochloride, we titrate 0.67 SH at pH 9, at pH 7, or down to pH 2. With mercury chloride we use a rotated mercury pool which is extremely simple to manipulate. If you make dialysis experiments, or quite generally, you might consider using mercury instead of silver because the mercury mercaptide is ever so much more stable. If you use mercury you might end up with that 0.67 of mercury per molecule of albumin.

MAZUR: Do I understand you correctly in saying that in the treatment with iodoacetamide you allowed the solutions to stay overnight?

JENSEN: We find this convenient. The iodoacetamide reaction is not instantaneous. It is an organic reaction with the reactants present in low concentration, so we like to give it a few hours. We usually put it away at room temperature in the evening, and in the morning the sulfhydryl is gone as evidenced by amperometric titration.

MAZUR: Our experience has been that if it is going to react with an —SH group, iodoacetamide does so in the first ten minutes; after that it continues to react at a slower rate, a linear rate which appears to be a nonspecific binding. This sort of reaction with iodoacetic acid was pointed out many years ago by Michaelis. I wonder in using the silver titration, where you add the silver and make your observation in a rather short period of time, if it is fair to assume that you are reacting with the same groups as you do when you are using iodoacetamide which has been in contact with the protein for so many hours?

JENSEN: The fact that iodoacetamide eliminates the sulfhydryl titer on amperometric titration and knocks out just one mole of silver binding suggests that the same chemical group in the protein is reacting with both reagents. Of course, amino groups can also react with iodoacetamide, but more slowly than sulfhydryl. Undoubtedly we lose a little of iodoacetamide to side reactions with amino groups, and also probably some due to a very slow hydrolysis in the water. If we add just one mole of iodoacetamide and do not let it stand for at least several hours, we will still have sulf-

hydryl there, or at least we have something that titrates in the amperometric titration.

FRAENKEL-CONRAT: I feel I have reached the stage of my life where I have to play the role of the old-timer. This meeting reminds me that there was a period in sulfhydryl research before Benesch and Boyer. At that time Greenstein determined the sulfhydryl of serum albumin by porphyrindin titration and he found that in urea there was none detectable, but in high concentration of guanidine one could titrate about 2 moles. That was the highest figure. We used that lots of times as a standard that our reagents were all right. Here we have another reagent giving a higher value, and if guanidine is presumed to break secondary valences such as hydrogen bonds, then your picture would not fit in with those observations. However, your picture did not really say whether you intended the secondary linkage of the sulfur to be a primary covalent linkage, or a secondary one that could be broken by denaturation alone. I hope you will tell us a little more, if you can be more specific, because this is the key question. We know that the sulfhydryl is in some way bonded, but the question is one of primary or secondary linkage.

JENSEN: If I did not specifically state what it is, it is because I do not know exactly what it is. We know it must be reversible because it can be reversed by the reaction with iodoacetamide or with a sufficient concentration of silver ions. My feeling is that whatever the bond is, it is not an irreversible covalent linkage, but nonetheless it is a very tight one. Masking to me at least is a steric phenomenon, whereas association of this kind is an equilibrium phenomenon and, therefore, different from the concept of masking.

BENESCH: I would like to add further to the confusion. Even in whole serum, the —SH titer increases in urea. If the amperometric silver titration of a serum is x, then the same serum is found to have $\frac{3}{2}\ x$ —SH groups in 8 M urea. This is significant because 90% of the SH is in the albumin fraction. The fact that one does not get this one-third increase with mercury as Dr. Kolthoff mentioned, or with methyl mercury (R. B. Simpson and H. A. Saroff, *J. Am. Chem. Soc.* **80**, 2021, 1958) might serve as an indication as to what the nature of this linkage might be. It is a linkage which can evidently be broken by silver in the presence of urea, but which cannot be broken by mercury in the presence of denaturing agents. I believe that the disulfide dye used by Dr. Klotz also does not show up the whole —SH group. Finally, I would like to emphasize that to obtain a whole —SH group per mole of serum albumin in the presence of urea by amperometric titration it is necessary to take very stringent precautions to have everything free of metals. If you have any metal traces present, it is very difficult to go up to one mole SH per mole.

JENSEN: That is certainly true. I will agree with that. One often gets commercial samples of albumin that titrate low in sulfhydryl, as low as 0.5 groups per molecule, simply because they have metal ions already there.

BENESCH: I would like to mention another point which Dr. Karush, who unfortunately could not be here, brought to my attention. He has observed, as many of us I think have, that sulfhydryl groups disappear in the presence of urea. We have explained this away with the rather facile explanation of oxidation. He has done experiments in the complete absence of oxygen, and even there the —SH groups disappear. He has proved the mechanism of this. Apparently even highly purified urea breaks down quite appreciably to cyanate which reacts with —SH groups to form —SCN.

JENSEN: I would like to ask one question myself of whomever may know the answer regarding mercaptalbumin. Suppose one titrates isolated mercaptalbumin amperometrically, what does one find? Do you know this, Dr. Benesch?

BENESCH: I am sorry, I do not.

JENSEN: If one takes mercaptalbumin regenerated again from the mercury dimer and titrates this amperometrically, either with or without urea, does this now show one sulfhydryl group, or does it titrate 0.7 of a sulfhydryl? Maybe Dr. Edsall knows.

EDSALL: Yes. We have done that and we get one sulfhydryl per molecule in water.

JENSEN: After you put it back in water it titrates one?

EDSALL: Yes. As regards Dr. Benesch's findings, if you can titrate in total albumin one sulfhydryl per mole in 8 *M* urea, the fact still remains that there are two kinds of molecules. On the one hand there is mercaptalbumin in which the sulfhydryl (although I agree with you that it is probably more or less masked) still readily reacts with —SH reagents in water. On the other hand there is the nonmercaptalbumin which has a latent sulfhydryl, but one which must be considerably more masked since it only becomes reactive in strong urea solutions.