therefore could not be incorporated into new DNA as large polynucleotides.

There are several other possible explanations for the differences observed between the two DNA's. The first is that real sequence differences are present in a few places and that they become distinguishable when larger polynucleotides are isolated. Another explanation would be that the fractions with high specific activities are phage DNA precursors isolated together with phage DNA from the infected bacterial cell but not present in viable phage. However, this would not explain the isolation of polynucleotides with very low specific activities. A third possibility is that phage DNA and the proflavin system DNA are made up of several polymers but the amounts of these polymers in the two systems are different although they have the same structure. Whichever possibility is correct, phage DNA and DNA isolated from proflavin-inhibited, phage-infected *E. coli* are not the same.

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The Disulfide Bonds of Human Serum Albumin and Bovine γ-Globulin[1]

BY GABOR MARKUS AND FRED KARUSH

RECEIVED JUNE 28, 1956

The structural role of disulfide bonds in human serum albumin (HSA) and bovine γ-globulin (BγG) has been evaluated by investigating the conditions of their reduction and its effect on the optical rotation and viscosity of the proteins. Reduction was effected with β-mercaptoethylamine HCl with and without sodium decyl sulfate. HSA has one free sulfhydryl group and 17 disulfide bonds, only one of which is reduced in the absence of detergent. Complete reduction of the disulfide bonds in the presence of detergent increases the reduced viscosity indicating disorganization of the tertiary structure. The concomitant large drop in the levorotation is interpreted as a gain in secondary structure. BγG has also one sulfhydryl group and 17 disulfide bonds, 5 of which can be reduced in the absence of detergent. Reduction of these 5 bonds does not change the viscosity and the optical rotation of this protein in the neutral *p*H range. Complete reduction in the presence of detergent causes little change in these properties beyond that brought about by the detergent alone. It is concluded that in HSA the disulfide bonds provide cross-links between distant portions of the polypeptide chain which stabilize the tertiary structure, and also prevent the maximum degree of secondary structure. Reduction releases the strain imposed by these bonds and permits the molecule to attain a higher degree of secondary structure with the accompanying loss of the original tertiary organization. In BγG, on the other hand, disulfide bonds contribute little to the cross-linking within the molecule as shown by the constancy of the high viscosity and levorotation after complete reduction in the presence of detergent. The bearing of these results on the problem of the intrinsic stability of α-helical structures in aqueous solution is considered.

During the past few years the α-helix of Pauling and Corey[2] has received increasing acceptance as the common and basic pattern for the organization of soluble proteins. This development has brought forth the recognition that the analysis of the structural organization of the protein molecule requires a conceptual distinction, as well as a corresponding experimental one, between secondary and tertiary structures.[3,4] The secondary structure arises from the stabilization achieved by the formation of hydrogen bonds between peptide groups relatively close to each other along the polypeptide chain. In the case of the α-helix each group is bonded to the third one beyond it. The tertiary structure emerges as a result of the interactions between the side chains of the amino acid residues. It is clear that the existence of the secondary structure will limit the tertiary organization. However, we know very little of the extent of their mutual dependence. It may be the case that the most stable tertiary structure precludes the full utilization of the peptide groups for intrahelical hydrogen bonding. It is quite possible, too, that for some proteins the integrity of the secondary structure can be maintained only by the additional stability provided by the tertiary interactions.

Among the various possible side chain interactions, the disulfide bond would be expected to be of particular importance. It appears to be the only commonly occurring covalent bond between side chains and it can provide a degree of stabilization of the tertiary structure far beyond that of any other interaction. Since this bond can be formed readily and specifically under physiological conditions and during the last stages of protein synthesis, it is well suited for the formation and stabilization of the selective configurations associated with the biological specificity of proteins. That the disulfide bond may play such a role is indicated by the fact that reduction causes the loss of biological activity of insulin[5] and of crotoxin.[6]

This paper describes the results of our investigations of the disulfide bonds of human serum albumin and bovine γ-globulin. We have sought to evaluate the structural role of these bonds in these two different types of proteins. Primarily, this has been done by relating the extent of reduction to changes in the specific rotation and reduced viscosity of the proteins. The results have been interpreted in terms of their secondary and tertiary structures.

(1) These studies were aided by a grant from the National Science Foundation and by a research grant (H-869) from the National Heart Institute of the National Institutes of Health, Public Health Service.

(2) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci.*, **37**, 205 (1951).

(3) K. U. Linderstrøm-Lang, Lane Medical Lectures, "Proteins and Enzymes," Stanford University Press, Stanford, California, 1952, p. 58.

(4) R. Lumry and H. Eyring, *J. Phys. Chem.*, **58**, 110 (1954).

(5) E. S. G. Barron, Z. B. Miller and J. Meyer, *Biochem. J.*, **41**, 78 (1947).

(6) K. H. Slotta and H. Fraenkel-Conrat, *Ber.*, **71**, 264 (1938).

Experimental

Materials.—Human serum albumin (HSA) was contributed by Cutter Laboratories and was 99% pure by electrophoretic analysis. Bovine γ-globulin (BγG) (Lot. No. P30012) was obtained from Armour Laboratories, as Fraction II from bovine plasma; 5 or 10% stock solutions were made up in redistilled water, and were kept frozen when not in use. The concentration of each stock solution was determined by Micro-Kjeldahl analysis taking 16.0% as the nitrogen content of both proteins. The reducing agent, β-mercaptoethylamine HCl (MEA), was obtained from Evans Chemetics, Inc., and was recrystallized from ethanol. Five-tenths M stock solutions were found stable over periods of several months if kept frozen. Sodium decyl sulfate (SDS) was synthesized according to Dreger, *et al.*[7] It was recrystallized twice from *n*-butyl alcohol.[8]

Reduction of Disulfide Bonds.—The solution contained protein in a final concentration of 1 or 2% and MEA in a range from 2.5×10^{-3} M to 2.0×10^{-1} M. Where sodium decyl sulfate was added, its final concentration was 0.1 or 0.2 M. The *p*H was adjusted to 7.0 (BγG) or 7.4 (HSA) before the solutions were brought to volume in 10-ml. flasks. In some experiments phosphate buffer of indicated ionic concentration was added to give a *p*H of 7.4. The solutions were incubated at room temperature for 1 hour before the removal of the reducing agent. This period was found sufficient to establish equilibrium. After incubation the *p*H of the solutions was adjusted to 5.0 to minimize the reversal of the reaction during removal of the MEA by the resin by converting all of the sulfhydryl groups to the undissociated form. The removal of MEA would also be facilitated thereby, since at this *p*H all the MEA molecules are positively charged. The solutions were kept at *p*H 5.0 up to the time of the SH-determination (10–30 minutes) since it was found that standing at *p*H 7.0 or higher causes rapid partial reoxidation.

Removal of MEA after Reduction.—This was accomplished by passage through columns of Dowex-50 (on the Na^+ cycle). Before use, the resin was thoroughly washed with distilled water. Each 10-ml. protein sample (1 or 2% protein) was passed through an individual column, 56 cm. × 0.6 cm. A rate of flow of about 0.15–0.20 ml./min. was found effective for the removal of 99.99% of the original amount of MEA when the MEA was passed through the column without protein. The MEA concentration in the effluent was measured by the spectrophotometric method of Boyer.[9]

Determination of Protein SH-groups.—In the early stages of this study SH content was determined by spectrophotometric titration with *p*-chloromercuribenzoic acid.[9] This method gave reliable and consistent results for high SH contents, but low protein SH contents were difficult to evaluate due to the high non-specific absorption of the protein in the ultraviolet region. Oxidation with ferricyanide[10] was sensitive enough but the results showed considerable variation and this method was therefore abandoned. The results here reported were obtained by amperometric titration with $AgNO_3$ in tris buffer (tris-(hydroxymethyl)-aminomethane) according to Benesch, Lardy and Benesch.[11] This procedure proved highly sensitive and specific, and the results were satisfactorily reproducible. For the details of this method reference is made to the original publication. Protein samples were diluted to give SH concentrations in the range of 1×10^{-3} M to 3×10^{-3} M. Sodium Lorol sulfate (du Pont) in a final concentration of 1% was added to the titrating mixture to increase the reactivity of the SH groups. It was found unnecessary to purify this material. All the other chemicals employed in the titration procedure were reagent grade and were dissolved in demineralized water.

In the few experiments where it was desirable to minimize reoxidation of the reduced disulfide bonds of albumin, iodoacetamide was added in a final concentration of 4×10^{-3} M after removal of the MEA. The protein concentration at this point was always less than 0.8%.

Optical rotations were measured in a Bellingham and Stanley polarimeter, graduated to 0.01 of a degree, using a sodium vapor lamp and a 100 or 200 mm. cell at room temperature. Viscosity measurements were made in Oswald–Fenske viscosimeters in a 25° water-bath. The blanks used for the calculation of reduced viscosities differed from the samples only by the absence of protein.

Since passage of solutions through resin columns caused some dilution, the protein concentrations of individual samples had to be determined. In the case of HSA the biuret reaction was employed.[12] The absorption of samples containing approximately 0.1% protein was measured at λ550 mμ, 30 minutes after the solutions were made up. Slight changes in the specific absorption due to aging of the reagent were compensated for by using a protein standard solution of known concentration. To avoid the effect of free SH groups on the biuret color, iodoacetamide was added to the protein sample taken for the analysis in a final concentration of 0.01 M 15 minutes before the addition of the biuret reagent. The concentration of BγG samples was determined by their absorption in 0.1 N NaOH at λ291 mμ 2 hours after the addition of the alkali using $\epsilon\lambda_{291}^{1\%}$ 15.2. The presence of detergent did not affect the absorption curve of this protein, whereas a marked shift was noticed in the case of HSA. The molecular weight of HSA was taken as 65,000, that of BγG as 156,000.

Results

A. Human Serum Albumin. Cysteine and Cystine Content.—Unreduced serum albumin contains 1.0 mole SH/65,000 g., when the titration is carried out in 1% sodium Lorol sulfate. This is in agreement with the recent findings of Benesch, *et al.*[11] Reduction of HSA with MEA up to 0.2 M, in the absence of detergent, yields only 2.8 SH groups per mole of albumin (Fig. 1, curve SH I), *i.e.*, only 0.9 disulfide bonds are split. Addition of sodium decyl sulfate in 0.2 M concentration greatly increases the reactivity of disulfide bonds (Fig. 1, curve SH II). At 0.1 M MEA a total of 35 SH groups is obtained. This figure agrees with the result obtained by amino acid analysis for cysteine and cystine/2.[13] In this connection it should be pointed out that in order to obtain maximum and reproducible values, the solutions should remain at *p*H 5 up to the time of titration. If they are adjusted to *p*H 7.4 and left standing, reoxidation of some of the SH groups occurs rapidly.

Viscosity and Optical Rotation.—The reduced viscosity of HSA at *p*H 7.4 and 25° is 0.039 (0.9% protein). In the absence of detergent the viscosity remains unchanged on the addition of MEA up to 0.2 M. The specific rotation of the HSA at *p*H 7.4 is −64.0 ± 0.5°, and shows the same constancy with respect to the MEA concentration. The reduction of 0.9 disulfide bond therefore does not measurably change these physical properties. Addition of 0.2 M sodium decyl sulfate to the unreduced protein causes a considerable change in both of these properties. The reduced viscosity increases to 0.225 and the specific rotation changes to −72.0°. Figure 1 shows the changes in viscosity and optical rotation as a function of the initial concentration of the reducing agent in 0.2 M SDS. Curve a shows the reduced viscosity measured in the presence of MEA. The large increase at higher concentrations suggests that the curve is the

(7) E. E. Dreger, G. I. Keim, G. D. Miles, L. Shedlovsky and J. Ross, *Ind. Eng. Chem.*, **36**, 610 (1944).

(8) The authors are indebted to Mr. Robert Marks for the synthesis and purification of this material.

(9) P. D. Boyer, THIS JOURNAL, **76**, 4331 (1954).

(10) E. S. G. Barron, *Advances in Enzymol.*, **11**, 201 (1951).

(11) R. E. Benesch, H. A. Lardy and R. Benesch, *J. Biol. Chem.*, **216**, 663 (1955).

(12) The biuret reagent consisted of 0.0100 M $CuSO_4$, 0.0040 M glycerol and 0.50 M Na_3PO_4. It was diluted 2-fold in the reaction mixture.

(13) G. R. Tristram, "The Proteins," ed. by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, IA, p. 215.

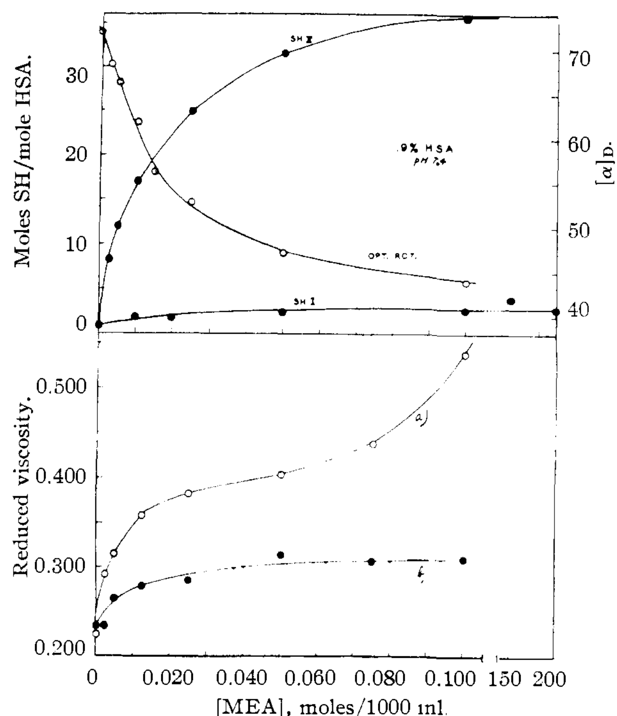

Fig. 1.—Reduction, specific rotation and reduced viscosity of HSA as a function of MEA concentration: curve SH I, reduction of disulfide bonds in the absence of detergent and added salt; curve SH II, reduction in 0.2 *M* SDS, no added salt. Curve opt. rot., specific rotation in 0.2 *M* SDS, and variable concentration of MEA, no added salt. Curve a, reduced viscosity in the presence of variable MEA and 0.2 *M* SDS, 0.05 *M* NaCl and 0.05 *M* phosphate buffer; curve b, reduced viscosity after exchange of MEA for NaCl, in 4 $\times$ 10^{-3} *M* iodoacetamide and 0.2 *M* SDS. Added salts as in curve a.

resultant of two processes. One of these is associated with the reduction of protein disulfide bonds and is independent of the continued presence of the reducing agent. This can be demonstrated by measuring the viscosity after the removal of MEA (curve b). (Oxidation of the liberated sulfhydryl was minimized by the addition of iodoacetamide.) This curve shows only the effect of the splitting of disulfide bonds and points up the role these bonds play in the stabilization of tertiary structure. The nature of the other process is not clear; it is probably the result of an interaction between MEA and the detergent. Since the effect is not cancelled out by correction for the blank containing these two reagents (reduced viscosity), the presence of the albumin must contribute to the increase of the viscosity. Negative results with 2-aminoethanol which differs from MEA only by the presence of a hydroxyl group instead of the sulfhydryl group, points to the importance of the latter group in the production of this effect. In this experiment 2-aminoethanol was added to the fully reduced protein solution containing iodoacetamide. That the SH groups of albumin play no part in this effect was shown by the return of the high reduced viscosity when MEA was restored to the iodoacetamide-treated albumin in the original concentration.

Figure 1 also shows the optical rotation of HSA reduced in the presence of 0.2 *M* SDS and measured in the presence of both detergent and reducing agent. The optical rotation changes very little on removal of the reducing agent and addition of iodoacetamide. For example, HSA reduced with 0.1 *M* MEA at *p*H 7.4 in the presence of 0.1 *M* SDS gave $[\alpha]_D$ $-43°$. On adjustment to *p*H 5.0, removal of MEA with the cationic resin column, reaction with iodoacetamide and return of the *p*H to 7.4, $[\alpha]_D$ was found to be $-45°$. The extent of the change in optical rotation is dependent on the number of disulfide bonds reduced. The striking feature of this behavior is the inverse relation between levorotation and SH content. Departure from the native state of proteins is usually accompanied by an increase in levorotation. In our case the levorotation decreased from 72 to 44°, *i.e.*, far beyond the 64° characteristic for the unreduced protein in the absence of detergent. The opposite behavior of viscosity and optical rotation here clearly indicates that these two measurements reflect different aspects of the structural change which was caused by the reduction of disulfide bonds. It will be pointed out in the discussion, that the combined use of these two measurements permits a distinction between changes in the secondary and the tertiary structures.

B. Bovine γ-Globulin. **Cysteine and Cystine Content.**—Like HSA, BγG has 1.0 free SH group/mole accessible to silver ions in the presence of 1% sodium Lorol sulfate. Figure 2 curve a shows the

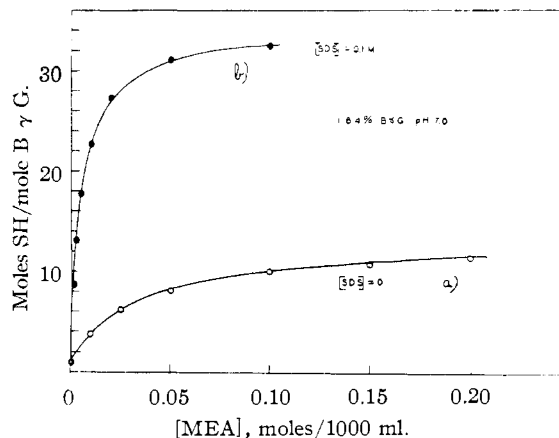

Fig. 2.—Reduction of BγG: curve a, without detergent; curve b, in 0.1 *M* SDS. No added salt.

number of sulfhydryl groups liberated as a function of the initial concentration of the reducing agent in the absence of detergent. The curve approaches the value of 11 SH groups showing that 5 disulfide bonds can be broken without the aid of detergent. This is in marked contrast to the 0.9 disulfide bonds split in HSA under similar circumstances. Addition of 0.1 *M* sodium decyl sulfate renders all disulfide bonds readily accessible to reduction (curve b). In separate experiments, using 0.2 *M* decyl sulfate a maximum value of 34.6 SH groups was obtained, *i.e.*, about 17 disulfide bonds were reduced. Amino acid analysis[14] yields a total of

(14) W. L. Hughes, in "The Proteins," ed. by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, IIB, p. 712.

37.6 cysteine and cystine/2 residues per 156,000 grams, corresponding to 18 disulfide bonds. Since γ-globulin is not a homogeneous protein, such discrepancies may arise from differences in the preparation and purification of the material.

Viscosity and Optical Rotation.—In the absence of detergent neither of these properties is affected by treatment with MEA at *p*H 7.0 (Fig. 3). The addition of SDS in 0.1 *M* concentration establishes a higher initial reduced viscosity. The rise in viscosity upon addition of the reducing agent up to 0.05 *M* is very slight, although at this concentration 15 out of the 17 disulfide bonds have been broken. Beyond this concentration there follows a time-dependent increase in the reduced viscosity which is accompanied by increasing turbidity of the solutions and is, therefore, due to aggregation. It is noted that in the case of HSA, a similar viscosity increase at higher MEA concentrations was not accompanied by turbidity and was independent of time. Extrapolation of the values to zero time shows only a slight increase in viscosity. A possible explanation of aggregation at higher MEA concentrations may be sought in the interaction between reducing agent and detergent. This interaction would decrease the number of detergent molecules bound to the protein and render the altered molecules less soluble.

The levorotation increases upon addition of the detergent from −45° to −61°. Reduction of disulfide bonds causes only a slight change (3°) in marked contrast to the large drop noted in HSA (28°). It is interesting to note that the optical rotations, in spite of increasing turbidity at the higher MEA concentrations, were independent of time, while the viscosity was not, suggesting that the aggregation reaction did not involve any further change in the structure of the protein molecule.

Further information about the stabilizing role of the disulfide bonds of BγG was obtained by exposure of the reduced protein to acid *p*H's. Curve a of Fig. 4 shows the constancy of optical rotation of BγG reduced with increasing amounts of MEA at *p*H 7.0 without detergent. The measurement of the optical rotation was done after adjustment to *p*H 5.0. If these samples are subsequently incubated at *p*H 3.0 for 60 minutes and their rotation measured (curve b) there is a large increase noted in all samples. The progressive reduction of up to 5 disulfide bonds substantially increases the levorotation at *p*H 3.0. When after 60 minutes the *p*H is readjusted to 5.0, the levorotation decreases by almost the same amount in all samples (6–7°). The irreversible rotatory change, however, caused by the low *p*H increases from 12% in the unreduced sample, to 62% in the sample treated with 0.15 *M* MEA.

Discussion

Reduction of HSA without Detergent.—The failure to reduce more than one disulfide bond with an overwhelming excess of the reducing agent (0.2 *M* MEA) may be accounted for in two ways. Either the disulfide bonds are located within the protein molecule so as to be inaccessible to MEA or the native protein gains so much in stability

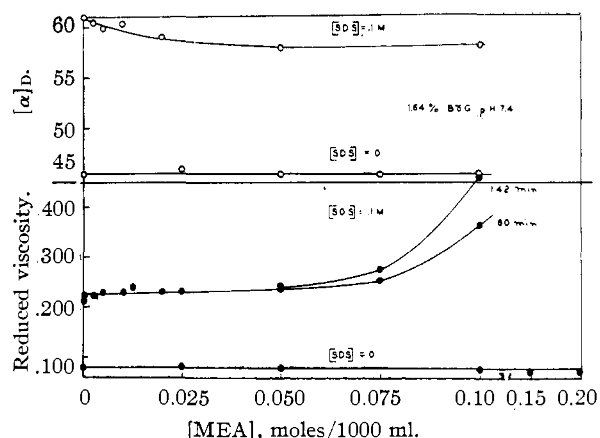

Fig. 3.—Dependence of the specific rotation and the reduced viscosity of BγG on the concentration of MEA, without SDS and in 0.1 *M* SDS. All solutions contain 0.05 *M* NaCl and 0.05 *M* phosphate buffer.

from the free energy difference between the oxidized and reduced forms that the equilibrium constant for the reaction with MEA is very much less than one. Our results do not allow us to choose between these alternatives. In any case this non-reactivity is another manifestation of the difference between serum albumin and γ-globulin in the character of their disulfide groups.

Reduction of HSA with Detergent.—The striking changes observed in the properties of extensively reduced albumin points to the important role the disulfide bond plays in affecting both the secondary and tertiary organization of this protein. The reduction of the disulfide bonds of HSA by MEA appears to be a reversible reaction, with respect to protein disulfide formation, as indicated by the dependence of the extent of reduction on the concentration of MEA. This conclusion is supported by the observation that the reduction with excess MEA is independent of the incubation period beyond one hour. The role of the detergent in this reaction appears to be that of rendering the protein disulfide groups accessible to the reducing agent. This effect is probably brought about by a swelling of the protein molecule as suggested by the fact that 0.2 *M* detergent increases the reduced viscosity from 0.039 to 0.225.

The presence of the detergent does not, however, allow as ready a reduction as is observed with simple disulfides. In the latter case it is found, *e.g.*, in the reaction between cystine and thioglycolic acid, that the equilibrium constant is of the order of one.[15] From the fact that an initial concentration of 0.01 *M* MEA is required to bring about reduction of one-half of the disulfide groups it may be estimated that the corresponding constant for the protein–MEA reaction is at least an order of magnitude smaller.

The further increase in the reduced viscosity which is observed on reduction of the protein, from 0.235 to 0.310, suggests that some of the disulfide bonds serve to link together sulfur atoms which are separated from each other by more than a few

(15) I. M. Kolthoff, W. Stricks and R. Ç. Kapoor, THIS JOURNAL, **77**, 4733 (1955).

peptide bonds along the single polypeptide chain of the protein. This observation is analogous to that reported by Frensdorff, Watson and Kauzmann.[16] They found that the intrinsic viscosity of bovine serum albumin at 30° in 8 M urea and pH 10 is increased from 0.2 to 0.5 when 0.02 M cysteine is present. Our results serve to confirm their interpretation of the cross linking role played by the disulfide bonds of serum albumin.

The unusual behavior of the optical rotation of the reduced protein provides a significant insight into the secondary structure of both the native and reduced molecule. We interpret the value of −44° for the specific rotation of the fully reduced protein compared to −64° for the native form to mean that cleavage of the disulfide bonds allows the molecule to form spontaneously additional intrahelical hydrogen bonds. That is, the molecule now assumes a secondary organization closer to the ideal α-helix than that which existed in the native molecule. It follows, therefore, that the disulfide linkages of the latter preclude for a considerable number of >N–H groups the requisite closeness of approach to >C═O groups required for the formation of a stable α-helix. Such a perturbing role of the disulfide bonds has been suggested by Frensdorff, Watson and Kauzmann[16] as the explanation for the comparative ease of urea denaturation of serum albumin.

It is to be emphasized that the reduction of the levorotation of the reduced protein is accompanied by an increase in viscosity. Whereas the value for the reduced viscosity falls in the range associated with the denaturation of globular proteins, the specific rotation, on the other hand, is in the range observed for native globular proteins. It is clear, therefore, that a rather sharp distinction can be made experimentally, in this case, between the secondary and tertiary structures. The combination of the detergent with the reduced protein very likely gives rise to a highly asymmetric molecule, effectively preventing the attractive intramolecular side-chain interactions which are required for the tertiary organization. With respect to the secondary structure, however, the additional stabilization resulting from reduction is sufficient to render the helix stable in spite of the disrupting tendency of the electrostatic charge of the detergent.

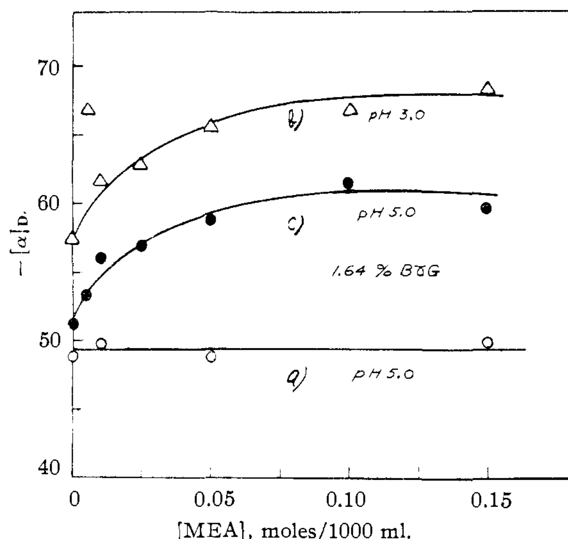

Fig. 4.—Effect of low pH on the specific rotation of BγG as a function of the MEA concentration. No salts added. Curve a, optical rotation at pH 5.0; curve b, same at pH 3.0; curve c, same after readjustment to pH 5.0.

These results have a significant bearing on the general problem of the intrinsic stability of hydrogen-bonded structures in aqueous solution.[17] We infer that for reduced albumin the structure is indeed stable at ordinary temperatures and that this may be true for many soluble proteins. Further interest attaches to this question in connection with the biosynthesis of proteins. If the polypeptide chain is synthesized in a more or less extended form, as would be expected if this process takes place on a nucleic acid template, then on release into the aqueous environment the chain must acquire the intrahelical hydrogen-bonded structure. Our results suggest that this change occurs spontaneously. Indeed, any other mechanism, for example, involving the intervention of an enzyme would seem to be ruled out. This we conclude on the grounds that the peptide hydrogen-bonding groups must be located in the core of the helix, surrounded by the side chains, and could not, therefore, participate in the equivalent of an enzyme–substrate complex. It is not precluded that side-chain interactions may be essential for the stability of the secondary structure. For purposes of biosynthesis it is necessary only that the probability of helix formation be sufficiently high to allow these interactions to organize the tertiary structure at the required rate. Finally, we may note that the formation of the tertiary structure may have the opposite effect, namely, loss of some secondary structure, as appears to be the case with serum albumin.

Reduction of BγG without Detergent.—Unlike HSA, treatment of BγG with MEA in the absence of detergent results in the splitting of about $^1/_3$ of the disulfide bonds. This fact points to the greater accessibility of this fraction of the disulfide linkages of BγG compared to those of HSA. It further allows us to distinguish clearly between two groups of disulfides in BγG in terms of their reactivity with respect to the presence of detergent.

Although the loss of the 5 disulfides does not change the specific rotation and the reduced viscosity at pH 7.0, we may conclude from the behavior of the reduced protein at pH 3.0 (Fig. 4), that these bonds do contribute to the stability of the native protein. It would appear that when these bonds remain intact the extent to which exposure to pH disorganizes the protein is sufficiently limited to allow reformation of the original secondary structure on readjustment of the pH to 5.0.

Reduction of BγG with Detergent.—The splitting of the additional 12 disulfide bonds which occurs in the presence of the detergent appears to have little effect on the structure of the protein molecule beyond that caused by the detergent alone. The

(16) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5167 (1953).

(17) For a detailed discussion of this problem see John A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **29**, 230 (1955).

large viscosity change brought about by the detergent itself, together with the constancy of viscosity on reduction suggests that these bonds contribute little to the cross-linking within the molecule. The absence of any large change in the optical rotation indicates that the two halves of each cystine residue are so located relative to each other that their disulfide bond does not prevent the assumption of secondary structure in its neighborhood.

Finally, we note the difference in the optical rotatory properties between fully reduced bovine γ-globulin and fully reduced human serum albumin, both in 0.1 *M* decyl sulfate. The albumin molecule, as we have already pointed out, seems under these conditions to acquire additional secondary structure relative to its native form, as revealed by the decrease of levorotation. The reduced γ-globulin molecule, on the other hand, appears to be less well organized in its secondary structure relative to the native protein. The disorganization of the unreduced molecule which combination with the detergent has caused persists in the fully reduced molecule. Since we have found that the elevated levorotation is retained after removal of the detergent, we may conclude that in this protein the stability of the secondary structure is dependent on the integrity of the tertiary organization.

PHILADELPHIA, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY SCHOOL OF MEDICINE]

Recovery of Native Bovine Serum Albumin after Precipitation with Trichloroacetic Acid and Solution in Organic Solvents[1]

BY GEORGE W. SCHWERT

RECEIVED JUNE 25, 1956

Bovine serum albumin, after precipitation with trichloroacetic acid, solution of the precipitate in alcohol or acetone, and removal of the solvent and trichloroacetic acid by dialysis, has been shown to be identical in sedimentation and electrophoretic behavior, in solubility, and crystallizability with untreated crystalline bovine serum albumin.

Recently, Levine[2] and Delaville, *et al.*,[3] have reported that human and bovine serum albumin, after precipitation with trichloroacetic acid (TCA), are soluble in organic solvents. Levine[2] found the TCA precipitate of bovine serum albumin (BSA) to be soluble only in polar solvents, such as methanol, ethanol and acetone, and also found that BSA which had been precipitated with perchloric acid or benzenesulfonic acid did not dissolve in organic solvents. Further, heat-denatured BSA was not soluble in organic solvents containing TCA and no precipitate appeared when alcoholic solutions of TCA-precipitated BSA were heated at 70° for 1 hour. Delaville, *et al.*,[3] in devising a method for the estimation of the albumin–globulin ratio of serum, made use of the fact that the TCA precipitate of serum globulins, unlike that of serum albumin, is insoluble in alcohol.

Although TCA is a powerful denaturing agent for most proteins, and although aqueous solutions of alcohol denature BSA unless the temperature is low,[4] the observations of Levine[2] raise the possibility that BSA is not denatured by treatment with TCA and solution of the precipitate in organic solvents.[5] The following experiments were carried out to test this hypothesis.

(1) Supported by grant G1161 from the National Science Foundation.

(2) S. Levine, *Arch. Biochem. Biophys.*, **50**, 515 (1954).

(3) M. Delaville, G. Delaville and J. Delaville, *Ann. pharm. franc.*, **12**, 109 (1954).

(4) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(5) This possibility is supported by unpublished observations, made by Dr. R. G. Parrish in this Laboratory in 1952, that alcohol solutions of TCA precipitated BSA remain clear and fluid at room temperature for a period of several days and set to a clear gel only after a period of weeks.

Experimental

Preparation of Protein.—Portions of two lots of Armour crystallized BSA were used for these experiments. One of these (Lot 20) had been stored at 5° for several years. The other (Lot P67704) was recently purchased. In a typical preparation 2 g. of BSA was dissolved in 50 ml. of water and precipitated by the addition of an equal volume of 10% TCA. The precipitate was collected by centrifugation at 1400 × *g* for 10 min., was suspended in 20 ml. of water and dissolved in 80 ml. of acetone or ethanol.[6] These steps were carried out at room temperature. The organic solvent solution was dialyzed for about 24 hours at 5° against several changes of distilled water,[7] the solution was pervaporated to a volume of about 50 ml. and again dialyzed for about 24 hours against several changes of distilled water at 5°. At this stage the protein solution is perfectly clear, but, as judged by a striking silkiness when the solution is stirred, contains a trace of suspended crystalline material.[8] The precipitate was removed by filtration and the protein was dried by lyophilization. BSA treated in this way is designated TCA–BSA.

Sedimentation Measurements.—Determinations of the sedimentation rates of BSA (Lot 20) and of TCA–BSA (Lot 20) were made in the Spinco Model E ultracentrifuge by techniques which have been described previously.[9] Measurements were made in *p*H 5.00 acetate buffer, Γ/2 0.2. Rotor temperature was assumed to increase linearly

(6) If organic solvents are added to the packed precipitate, it is difficult to suspend the precipitate fast enough to prevent the formation of particles of gel which dissolve very slowly. If, however, the precipitate is first suspended in a small volume of water, it dissolves readily when the organic solvent is added.

(7) If alcohol is used as the solvent for TCA–BSA, the protein precipitates during the dialysis and later redissolves. Dr. Joseph C. Rupp has pointed out that this precipitation can be avoided by using acetone as the solvent.

(8) These particles are barely resolved by the light microscope. The author is indebted to Dr. D. Gordon Sharp for electron micrographs which reveal the particles to be highly asymmetric parallelograms in plain view. The crystals are insoluble in water but readily soluble in ethanol. The ultraviolet absorption spectrum of a dilute solution in ethanol is characterized by a sharp maximum at 218 mμ. The nature of this material is being investigated.

(9) G. W. Schwert, *J. Biol. Chem.*, **179**, 655 (1949).