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Interactions of Proteins with Disulfide Compounds: Some Implications for Electron Transport in Proteins

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The reactions of serum albumin, β-lactoglobulin and ovalbumin with a disulfide compound have been studied by photometric titrations and by equilibrium dialysis methods. The thiols of ovalbumin react with the disulfide in a manner analogous to the reaction of small-molecule mercaptans. On the other hand, the -SH groups of serum albumin or β-lactoglobulin react with the disulfide to give mixed disulfides exclusively in a manner that has a stoichiometry and other features that are difficult to explain by any simple mechanism. It is suggested that the electrons which are transferred in this reaction (as well as in other oxidation–reduction reactions) are transported by a shift of an H^- through the hydration lattice of the protein molecule. The possible significance of such a mechanism in energy transport in biochemical systems is discussed.

Introduction

Past investigations of soluble protein complexes with small molecules have dealt largely with reversibly-bound substances. It has seemed likely that some further insight into protein structure and behavior might be obtained from a study of complexes formed through covalent bonds. A promising reaction in this connection is that between protein mercaptan groups and disulfides, since the reaction occurs readily at room temperature and since there exists a substantial background of quantitative information on the corresponding reaction in simpler systems.

This mercaptan interchange in proteins

$$\text{P-SH} + \text{R-S-S-R} \longrightarrow \text{P-S-S-R} + \text{R-SH} \quad (1)$$

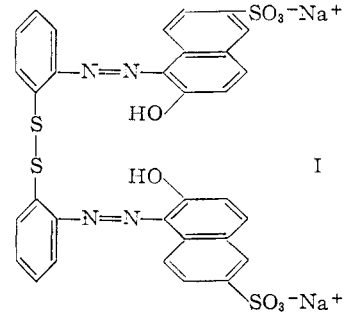
is specifically of interest as a possible mechanism for a variety of protein aggregation reactions. It was suggested originally by Huggins, Tapley and Jensen[1] as an explanation of the gelation of plasma albumin in urea. It has been proposed subsequently to account for cross-linking exchange phenomena in keratin,[2] wool,[3] ovalbumin[4] and fibrin.[5] Furthermore, from another viewpoint this interchange is essentially an oxidation–reduction reaction and hence its course in a protein environment may provide some clues as to the mechanism of protein participation in other oxidation–reduction reactions. It seems worthwhile, therefore, to study this interchange reaction directly.

In non-protein systems, mercaptan-disulfide interchange in small molecules has been investigated extensively, quantitative studies of the equilibria having been reported by Bersin and Steudel[6] and more recently by Kolthoff, Stricks and Kapoor,[7] by Fava, Iliceto and Camero,[8] and by Eldjarn and Pihl.[9] The results from these simple systems cannot be carried over directly to proteins, however, as is immediately evident from the fact that many proteins with available mercaptan groups give no evidence of intermolecular disulfide interchange under normal conditions, even though the reaction between small molecules is rapid.

Since it is difficult to examine the state of disulfide groups in proteins, but relatively simple to characterize the mercaptan group, we have investigated the reaction between protein sulfhydryl and externally-added, small-molecule disulfide. To follow the course of the reaction, a colored disulfide compound was used. Its spectrum when cleaved and bound to protein through an S–S linkage is appreciably different from that of the free disulfide molecule. Spectroscopic changes thus provided a convenient basis for following quantitatively the effects of various factors on the course of the reaction.

Results and Discussion

Stoichiometry of the Reaction.—It is important first to establish the stoichiometry of the reaction in order to determine whether the mole ratios are the same as when both reactants are small molecules. The colored disulfide used, the disodium salt of 2,2′-(2-hydroxy-6-sulfonaphthyl-1-azo)-diphenyl disulfide (I) (abbreviated as DSSD),



I

would be expected to react in either of two ways[10] with a protein containing one -SH

(1) C. Huggins, D. F. Tapley and E. V. Jensen, *Nature*, **167**, 592 (1951).

(2) A. M. Woodin, *Biochem. J.*, **57**, 99 (1954).

(3) R. W. Burley, *Nature*, **175**, 510 (1955).

(4) M. Halwer, THIS JOURNAL, **76**, 183 (1954).

(5) A. Loewy and J. T. Edsall, *J. Biol. Chem.*, **211**, 829 (1954).

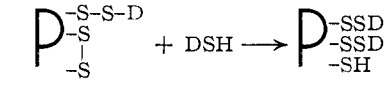
(6) T. Bersin and J. Steudel, *Ber.*, **17B**, 1015 (1938).

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

(8) A. Fava, A. Iliceto and E. Camero, *ibid.*, **79**, 833 (1957).

(9) L. Eldjarn and A. Pihl, *J. Biol. Chem.*, **225**, 499 (1957); THIS JOURNAL, **79**, 4589 (1957).

(10) An important possibility, as far as stoichiometric consequences are concerned, would be subsequent reduction of disulfide groups in the protein by the DSH produced in (2) or (3), followed by further reaction with D-S-S-D. Thus, following reaction 2 we could write



and the -SH groups produced could combine with more D–S–S–D. This reaction would be terminated when all the internal S–S groups of the protein had been converted to -S–S–D. The ratio of moles dye reacted to protein supplied would be very high, for serum albumin, for example, over 15. Even rough early experiments showed, however, that this kind of a chain reaction does not occur, neither in simple aqueous solution nor in urea solutions.

$$P\text{-}SH + D\text{-}S\text{-}S\text{-}D \longrightarrow P\text{-}S\text{-}S\text{-}D + D\text{-}SH \quad (2)$$

$$2\,P\text{-}SH + D\text{-}S\text{-}S\text{-}D \longrightarrow P\text{-}S\text{-}S\text{-}P + 2D\text{-}SH \quad (3)$$

Since the mole ratio of reacted dye to protein is so different in (2) and (3), a quantitative experiment should choose easily between them.

The spectrum of D–S–S–D in aqueous buffer has a peak at 515 mμ, that of P–S–S–D[11] at 490 mμ (Fig. 1). The change in absorption at a wave

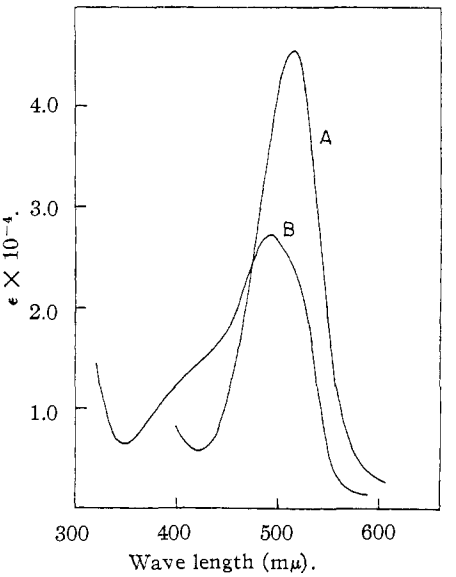


Fig. 1.—Absorption spectra of disulfide dye, DSSD: A, in acetate buffer *p*H 6; B, after reaction with bovine serum albumin in same buffer.

length, such as 530 mμ,[12] where the intensity of free *versus* reacted dye is very different, forms a convenient basis for measuring the extent of reaction. A typical titration curve, based on optical changes, is shown in Fig. 2. The end-point is quite sharp and reproducible.

For bovine serum albumin, with 0.67 SH group per mole of protein, the moles of DSSD per mole of protein at the end-point falls consistently in the range 0.33–0.37 (Table I). It seems clear, therefore, that in the reaction the mole ratio of protein thiol to dye disulfide is 2 and hence that equation 2 may be ruled out.

As a further check on the stoichiometry, an equilibrium dialysis experiment[13] also was carried out. The moles of bound dye per mole of serum albumin are plotted in Fig. 3. The binding rises steeply to 0.34 mole of dye and then breaks off into a slow rise characteristic of the reversible general binding of anionic dyes by serum albumin. The sharp break at 0.34 mole must correspond to the specific covalent binding of dye and presumably, therefore, to the reaction with the mercaptan group of the protein. Thus the dialysis experiments confirm the optical titration data in indicating a mole ratio of 2 for protein thiol to reacted dye.

(11) A corresponding change in spectrum is obtained when a simple mercaptan, RSH, reacts with DSSD to give R–S–S–D and H–S–D. Peaks at 490 mμ are obtained upon addition to DSSD of any one of the following: thioglycolic acid, cysteine, glutathione, thiomalic acid, dimercaptosuccinic acid.

(12) At *p*H 2.5, the absorption spectrum of DSSD is lowered markedly compared to higher *p*H's, probably because of micelle formation. At *p*H 2.5, the change in absorption at 420 mμ proved to be much more convenient for measuring the extent of reaction.

(13) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

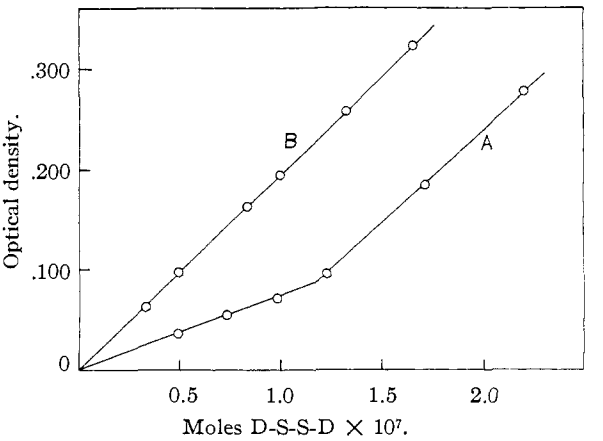


Fig. 2.—Photometric titration curves for disulfide dye, DSSD, and protein in 0.2 *M* acetate buffer *p*H 6.01, wave length, 530 mμ: A, bovine serum albumin, 0.1%; B, iodinated bovine serum albumin, 0.1%.

TABLE I

STOICHIOMETRY OF REACTION OF DISULFIDE DYE, DSSD, WITH BOVINE SERUM ALBUMIN[a] AT 25° FROM PHOTOMETRIC TITRATIONS

Conditions	Moles dye reacted per mole protein
0.2 *M* acetate buffer, *p*H 6	0.35 ± 0.02
.2 *M* acetate buffer, *p*H 6, iodinated protein	.00
.2 *M* acetate buffer, *p*H 6, deaerated	.37
.2 *M* acetate buffer, 0.01 *M* citrate, *p*H 6	.36
0.2 *M* NaCl, *p*H 6	.35
Water, *p*H 6	.35[b]
0.2 *M* NaCl, *p*H 4.9	.35
Water, *p*H 5	.35[b]
0.2 *M* acetate, *p*H 6, 1.0% protein	.35
.1 *M* citrate, *p*H 2.5	.40
.4 *M* acetate, *p*H 4.8	.36
.2 *M* acetate, *p*H 6	.35
.1 *M* tris-hydroxymethylaminomethane, *p*H 7.5	.35
.4 *M* glycine, *p*H 9.2	.36
.2 *M* acetate, *p*H 6, 2 *M* guanidine hydrochloride	.33
.2 *M* acetate, *p*H 6, 8 *M* urea	.20, 0.23, .25, 0.32
.2 *M* acetate, *p*H 6, 2.5 *M* glycine	.36
.2 *M* acetate, *p*H 6, 5 *M* β-alanine	.41
.2 *M* acetate, *p*H 6.1, 1.5 × 10^{-4} *M* propionaldehyde	.33
.2 *M* acetate, *p*H 6, 10^{-5} *M* styrene	.32
.2 *M* acetate, *p*H 6, satd. soln. styrene	.34
.2 *M* acetate, *p*H 6, 10^{-3} *M* dihydroxyphenylalanine	.33
.2 *M* acetate, *p*H 6, 10^{-3} *M* catechol	.31
.2 *M* acetate, *p*H 6, 10^{-3} *M* picric acid	.33
.2 *M* acetate, *p*H 6, 10^{-3} *M* tyrosine	.34

[a] The protein concentration was 0.1% in all cases except the one listed at 1.0%. [b] In these cases, the reaction required one hour. In all other entries, end-points were reached in a few minutes.

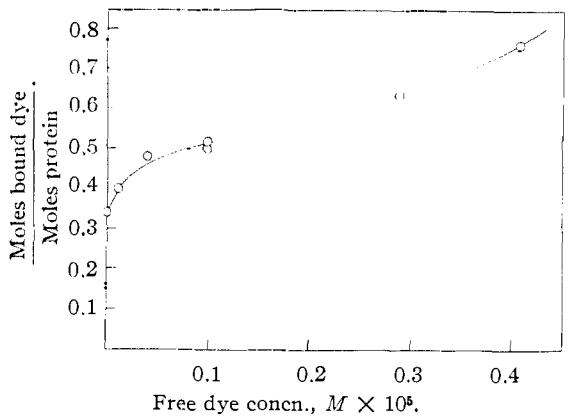


Fig. 3.—Binding of disulfide dye, DSSD, by 0.2% bovine serum albumin in acetate buffer, *p*H 6.00, at 25°.

Although both types of experiment fit well the stoichiometry of equation 3, it seemed prudent to use a chemical approach to assure one that the dye was really reacting with the mercaptan group of the protein. An optical titration was carried out, therefore, with iodinated bovine serum albumin, in which the –SH group has been oxidized.[14] As the results in Fig. 2 demonstrate, the titration of iodinated protein with dye gives no indication of a break.[15] The slope of the curve is the same as that for DSSD added to buffer alone. Clearly then, a protein in which the –SH has been removed shows no reaction with DSSD. The sharp break in the titration curve with unmodified bovine albumin must be, therefore, a measure of the –SH in this protein.

It is thus apparent immediately that serum albumin mercaptan behaves markedly differently from a small molecule mercaptan, RSH. In the latter case the work of several groups of investigators[6–9] shows that RSH does not exhibit a sharp stoichiometry in its reaction with disulfide compounds, R'SSR', but rather that two equilibria are involved, with equilibrium constants of order of magnitude near 0.1–10

$$RSH + R'SSR' \rightleftharpoons R'SSR + R'SH \quad (4)$$

$$R'SSR + RSH \rightleftharpoons RSSR + R'SH \quad (5)$$

In contrast, with the protein mercaptan, P–SH, no equilibrium is detectable,[16] the reaction going completely to the right. Why this should be so was not evident from equation 3, but did become apparent from further work.

Mechanism of the Reaction.—Equation 3, which fits the stoichiometry of the protein-DSSD reaction, implies that an albumin dimer is formed. Since two-thirds of the albumin has a mercaptan group, two-thirds should be in the form of dimer, with a molecular weight of about 130,000. In the ultracentrifuge it should be easy to distinguish the dimer from the residual monomer, since the two are not in equilibrium. It was surprising, therefore, to find that the sedimentation pattern of serum albumin after reaction with DSSD was not distinguishable from that of a fresh protein solution unexposed to dye (Fig. 4). Evidently the molecular weight of the protein is not changed in the manner implied by equation 3.

It was conceivable, however, that the dimer was actually formed but in such a way that the resultant molecule had a very asymmetric structure. In such a case its sedimentation constant would not differ appreciably from that of the monomer, despite the higher molecular weight of the former. Molecular weights were determined, therefore, both by osmotic pressure and sedimentation methods. The former gave values near 60,000, the latter 74,000. The osmotic pressure results are slightly lower than the value for pure monomeric protein. The sedimentation data are slightly high, perhaps because of the presence of a small amount of polymeric material in the initial serum albumin. From each of these results, nevertheless, one can rule out unequivocally the formation of P–S–S–P indicated by equation 3. We are thus led to the dilemma that molecular weight measurements show that equation 3 cannot be correct, at least as regards the products, and stoichiometric data rule out equation 2.

In connection with the ultracentrifugal experiments, it should be noted that at high speeds (60,000 r.p.m.) the dye sedimented with the protein. While no quantitative densitometry was carried out, visual examination of the ultracentrifuge screen during sedimentation gave no indication of any dye above the protein boundary (see also Fig. 4A). Thus it seems that essentially all of the dye is held by strong covalent bonds to serum albumin. This conclusion is reinforced by the equilibrium dialysis experiments (Fig. 3). The very steep initial rise of the binding curve and the sharp bend at 0.34 mole bound dye are characteristic of covalent irreversible binding, not of the relatively weak, reversible anion complexes of serum albumin.

These apparently contradictory stoichiometric and sedimentation data could be explained readily if atmospheric oxygen participated in the reaction

$$P\text{–}SH + DSSD \longrightarrow P\text{–}SSD + D\text{–}SH \quad (2)$$

$$D\text{–}SH + {}^1/_4O_2 \longrightarrow {}^1/_2DSSD + {}^1/_2H_2O \quad (6)$$

The net result of reactions 2 and 6 would be the disappearance of only $^1/_2$DSSD per mole of protein SH, since $^1/_2$DSSD is regenerated in step 6. If oxygen were involved, however, one would expect the stoichiometry of the reaction to change under anaerobic conditions, for the "regeneration" reaction of equation 6 could not occur. Mixing

(14) Attempts also were made to block the protein mercaptan with salyrgan, an organic mercurial. This substance slowed down the rate of reaction enormously but was unable to stop the reaction completely. Likewise the azomercurial 4-(*p*-dimethylaminobenzeneazo)-phenylmercuric acetate merely slowed down the reaction. On the other hand, acetylated serum albumin behaved like the iodinated protein.

(15) Comparable results were obtained with solutions of bovine albumin in the presence of 0.7 mole of quinone per mole of protein. As will be shown in a separate paper, quinone and the mercaptan in albumin react in a 1:1 mole ratio. After this reaction, the dye DSSD no longer undergoes any changes if added to the albumin–quinone adduct.

(16) One could argue, of course, that the difference in the protein reaction lies in the different disulfide used, the dye DSSD being very different from molecules like cystine used by Bersin and Steudel[6] and by Kolthoff, Stricks and Kapoor.[7] That this difference is not significant was shown by a number of experiments with DSSD and mercaptans such as cysteine and thioglycolic acid. The optical titration curves show a gradual change in slope, typical of a reaction near equilibrium.

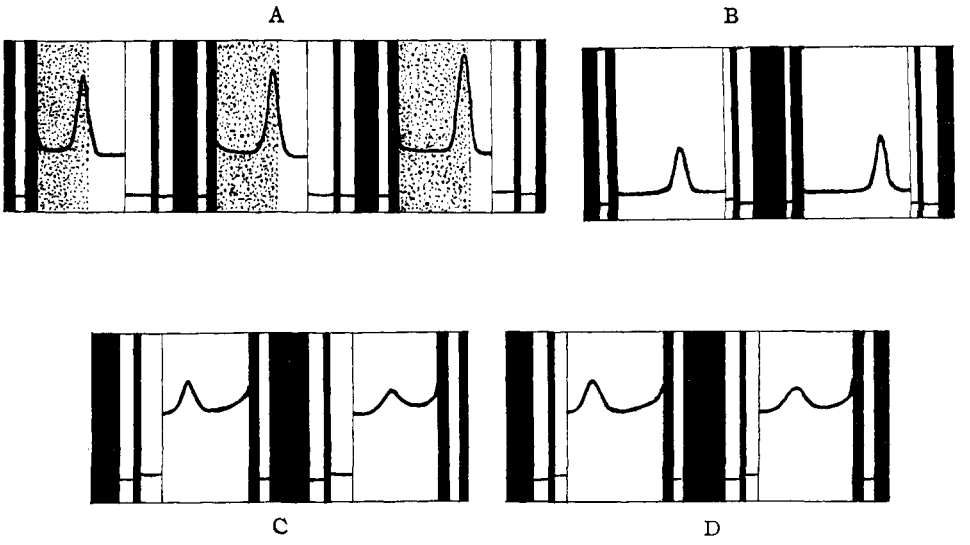


Fig. 4.—Tracings of schlieren patterns of: A, 1% bovine serum albumin mixed with DSSD in 3:1 mole ratio in acetate buffer, *p*H 6, at three different times to show movement of dye boundary with protein peak; B, bovine serum albumin alone in same buffer; C, 0.44% β-lactoglobulin mixed with DSSD in 1:1 mole ratio in acetate buffer, *p*H 6; D, β-lactoglobulin alone in same buffer. Sedimentation was in a Spinco ultracentrifuge, Model E, at 59,780 r.p.m.

experiments were carried out, therefore, in a specially designed two-legged inverted U-tube[17] which permitted one to remove air from each solution (protein and dye) by bubbling prepurified nitrogen through each leg before mixing. The optical titration curve under these conditions still showed a break at 0.37 mole DSSD per mole bovine albumin (Table I), in other words, at the same point as when oxygen is present. Clearly then oxygen does not participate in the reaction and equation 6 may be ruled out.

An alternative explanation of the observed behavior would be one in which a reducible metal takes the place of oxygen in equation 6. For example, with copper, one might write

$$\text{D–SH} + \text{Cu(II)} \longrightarrow {}^1/_2\text{DSSD} + \text{Cu(I)} + \text{H}^+ \quad (7)$$

Spectrochemical analysis[18] of serum albumin revealed, indeed, the presence of both copper and iron but the quantities present were below 0.005%. Hence for reaction 7 to contribute to the stoichiometry, the Cu(I) (or the Fe(II) if iron were involved) would have to be re-oxidized, *i.e.*, the metal would have to act essentially in a catalytic capacity. Such a reoxidation could conceivably occur, even in the absence of oxygen, by a reaction with S–S groups in the protein. To test this possibility the rate and stoichiometry were measured in the presence of 0.01 *M* citrate, a strong chelating agent for copper or iron. No detectable differences were observed (Table I). It seems unlikely, therefore, that a metal participates in the reaction.

The 2:1 stoichiometry of protein mercaptan to DSSD thus seems to arise from some characteristics of the reactants themselves. A possible explanation avoiding any outside agents would be the following pair of reactions

$$\text{P}\begin{matrix}\text{–SH}\\ \text{—S}\\ \text{—S}\end{matrix} + \text{D–S–S–D} \longrightarrow \text{P}\begin{matrix}\text{–S–S–D}\\ \text{—S}\\ \text{—S}\end{matrix} + \text{HS–D} \quad (8)$$

$$\text{P}\begin{matrix}\text{–SH}\\ \text{—S}\\ \text{—S}\end{matrix} + \text{HS–D} \longrightarrow \text{P}\begin{matrix}\text{–S–S–D}\\ \text{–SH}\\ \text{–SH}\end{matrix} \quad (9)[19]$$

The first of these is essentially equation 2, written in a more detailed fashion to point up the presence of disulfide, as well as mercaptan groups in the protein. Equation 9, however, contains a new feature, the assumption that a disulfide group of serum albumin participates in the reaction of D–SH with the protein mercaptan to give a second mixed protein–dye disulfide. These equations account for the observed stoichiometry, if it is assumed that reaction 9 is much faster than (8). Clearly also these equations provide for the formation only of protein monomers, not of dimers, in agreement with ultracentrifugal and osmotic pressure observations.

As written, equation 9 implies that the S–S group of the protein is near its –SH. This may indeed be the case with bovine serum albumin, for it has been shown previously that the behavior of copper–albumin complexes can be accounted for by a similar picture.[20] Nevertheless, this aspect of equation 9 should not be taken literally for, as will be shown below, other protein mercaptans also react with DSSD in a 2:1 ratio and it seems unlikely that all of these have mercaptan groups in the immediate

(17) G. Czerlinski, M.S. Dissertation, Northwestern University, 1955.

(18) The analyses were carried out by the Chicago Spectro Service Laboratory.

(19) To forestall any misunderstanding, it should be emphasized that the product of equation 9 cannot be $\text{P}\begin{matrix}\text{–SH}\\ \text{–SSD}\\ \text{–SH}\end{matrix}$ because in that case the original –SH of the protein would still be free, and consequently it should react with more DSSD until a 1:1 stoichiometry of protein mercaptan to DSSD has been attained.

(20) I. M. Klotz, J. M. Urquhart, T. A. Klotz and J. Ayers, THIS JOURNAL, 77, 1919 (1955).

vicinity of disulfides. Further consideration of the reaction 9 will be postponed, therefore, until observations with other proteins are described below.

Thiol Groups Left at Conclusion of Reaction.—Equations 8 and 9, as well as the previous reaction schemes other than the one involving oxygen, imply that mercaptan groups are still present even after the reaction of serum albumin with DSSD has ended. The fact that the reaction stops sharply when the initial protein –SH has reacted with $^1/_2$ mole of DSSD would require further that the mercaptan groups produced as a result of the reaction be "masked" since they are not accessible to reaction with the dye. Both of these conclusions have been substantiated by experiments with other reagents for mercaptans.

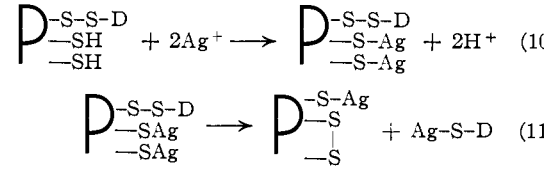
In studies with a colored azomercurial,[21] we have shown that hindered as well as accessible –SH groups (compare β-lactoglobulin and bovine serum albumin) bind this compound stoichiometrically. Therefore, the uptake of 4-(*p*-dimethylaminobenzeneazo)-phenylmercuric acetate was measured with untreated serum albumin and with a sample of protein exposed to enough DSSD to react with 75% of its mercaptan groups. The uptake of mercurial was 0.66 mole with the former protein, 0.63 mole with the latter. Clearly, approximately as much mercaptan is present at the conclusion of the reaction with DSSD as was in the protein at the outset.

The amperometric silver method of titrating mercaptan,[22] on the other hand, showed a marked difference in the nature of the –SH group present at the end of the reaction with dye. Bovine serum albumin reacts rapidly with silver ion, in tris-hydroxymethylaminomethane buffer (Fig. 5). On the other hand, amperometric titration of the protein–dye reaction product shows the typical pattern (Fig. 5) of a masked –SH group; silver ion is taken up, but very slowly.

It is pertinent to emphasize that in this protein–dye complex we have *produced* a masked sulfhydryl group, for in the original protein the mercaptan was readily accessible, at least to silver ion.[23] The newly-produced sulfhydryl group thus must be in a different environment than that present at the outset in serum albumin.

The very sharp end-point of the reaction between protein and DSSD can thus be understood readily on the basis of the modified nature of the thiol groups produced in the course of the reaction. Since these are hindered, as judged by silver titrations, it is no surprise that the DSSD molecules cannot penetrate either. Hence the reaction stops when all of the original, accessible –SH groups have reacted.

The masked –SH group produced in serum albumin after reaction with DSSD becomes readily accessible to Ag^+ in the presence of added denaturant. Amperometric titration in 8 *M* urea shows a rapid uptake of silver ion. The end-point obtained, 0.78 mole of sulfhydryl per mole of protein, is somewhat anomalous, however, for it corresponds neither to 0.68 found in the original protein in aqueous buffer nor to approximately 1.0 found for the original albumin placed in urea.[22] The significance of these results in urea may be obscured, however, by what appears to be cleavage of the P–S–S–D bonding in the presence of Ag^+ (or Hg^{++}), for during the titration of the yellow protein–dye complex a pink color slowly appears which is very similar to that obtained when Ag^+ is added to the dye in its mercaptan form, D–SH. Hence it appears likely that these reactions occur



(10)

(11)

It is quite possible, furthermore, that the D-S-Ag forms a ternary complex with serum albumin in which the metal acts as a bridge between the dye and the protein.[24] The net result of all of these reactions which seem to take place in urea solution could lead to a stoichiometry for uptake of Ag^+ which would be difficult to interpret. Nevertheless, it should be recalled that, despite ambiguities in the quantitative aspects of the silver titration in urea, the production (in the reaction with DSSD) of one mole of SH for every mole originally present in the protein has been clearly established by the assay of thiol with the azomercurial described above.

Influence of Denaturants.—It was our expectation that in the presence of a denaturant the extent of reaction of serum albumin with DSSD would be greatly increased since any mercaptan groups produced in the protein presumably would no longer be masked. It was a surprise, therefore, to find that in the presence of 8 *M* urea, less DSSD reacted per mole of serum albumin than in the absence of denaturant. It was also not possible to obtain as reproducible end-points in the presence of 8 *M* urea as in its absence; end-points varied[25] from 0.20 to 0.32 mole of DSSD (Table I).

Guanidine hydrochloride (2 *M*) did not change the stoichiometry of the protein–disulfide reaction (Table I).

In contrast, in the presence of 1% sodium dodecyl sulfate, the end-point of the reaction was slightly high, 0.41 mole DSSD per mole of albumin. The initial reaction leading to this end-point was rapid, being completed in less than two minutes. There was an indication of a succeeding very slow reaction which over a period of days led to slightly higher uptakes of dye. These trends were magnified in the presence of 3% dodecyl sulfate,[26] the

(21) M. G. Horowitz and I. M. Klotz, *Arch. Biochem. Biophys.*, **63**, 77 (1956).

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

(23) This result is in some respects similar to that of V. M. Ingram, *Biochem. J.*, **59**, 653 (1955), who found that after reaction of hemoglobin with mercury in an amount less than equimolar with the –SH content, uptake of silver ions was prevented.

(24) I. M. Klotz and W. C. L. Ming, THIS JOURNAL, **76**, 805 (1954).

(25) The low results may reflect in part a more rapid oxidation of the protein mercaptan groups by air in the presence of urea, or perhaps changes in state of aggregation of the protein in the solution with this denaturant. *Cf.* I. M. Kolthoff, A. Anastasi, W. Stricks, B. H. Tan and G. S. Deshmukh, *ibid.*, **79**, 5102 (1957).

(26) The detergent may be favoring slight hydrolysis of protein disulfide bonds. Amperometric silver titrations of solutions of bovine serum albumin which had been exposed to 3% dodecyl sulfate like-

end-point at two minutes being 0.52 mole DSSD and increasing slowly to 0.83 over a period of three days.

With none of these denaturants is there any indication of a true chain-like mercaptan–disulfide interchange[10] in which the protein thiols produced in reactions corresponding to equation 9 continue to react rapidly with DSSD, repeating equation 8, and continuing on with (9), etc. It seems clear that the –SH groups produced in the protein as a result of the disulfide interchange are different in nature from those initially present. Perhaps this difference is due to the fact that the new thiols occur as twins, that is, they have both been generated from the same parent S–S group (see equation 9) and hence remain in close association.

Comparison of Proteins.—The interaction of disulfide dye was examined with the proteins β-lactoglobulin and ovalbumin, in addition to serum albumin.

A marked difference was noted immediately in the rate of reaction of β-lactoglobulin as contrasted to serum albumin. With the latter, in the presence of salts, the reaction was completed in 1–2 minutes. With β-lactoglobulin, however, the uptake of dye at *p*H 6 was spread over many hours, or even days.[27] The slowness of the interaction between β-lactoglobulin and dye probably resides in a decrease in rate of equation 8. If the sulfhydryl group in this protein were hindered, one would expect a decrease in speed of its reaction with outside molecules. The existence of masked –SH groups in β-lactoglobulin is clearly evident in amperometric silver titrations. In aqueous buffer Ag^+ is taken up slowly and a fast titration gives a zero titer; only in urea do two thiols per protein molecule show up readily.

The speed of the reaction with β-lactoglobulin increases as the *p*H is raised. Experiments in acetate and in tris-hydroxymethylaminomethane buffers of *p*H 7 reached clear-cut end-points of 1.0–1.1 moles DSSD in less than a day. The reaction in glycine, *p*H 9.2, was complete in less than an hour. The increased rate with increasing *p*H is consistent with the general view that it is the S^- ion which is the reacting species in mercaptan–disulfide exchange reactions.[28]

It is pertinent to mention that the net sulfhydryl content of this system also remains constant during the reaction. In aqueous solution, β-lactoglobulin alone reveals no mercaptan groups in a fast amperometric titration. In the presence of 8 *M* urea, the protein titrates slightly over two moles of Ag^+. Likewise the β-lactoglobulin–dye complex does not take up Ag^+ rapidly in aqueous solution, but in urea consumes slightly over 2 moles. Thus there seem to be two masked –SH groups in native β-lactoglobulin and in the dye complex. It should be mentioned in this connection that the yellow color of the dye travels with the β-lactoglobulin in a sedimentation experiment at 60,000 r.p.m. in the Spinco ultracentrifuge; clearly a strong bond exists between dye and protein. Also, as with serum albumin, there was no evidence of dimers of β-lactoglobulin after reaction with the D–S–S–D (Fig. 4).

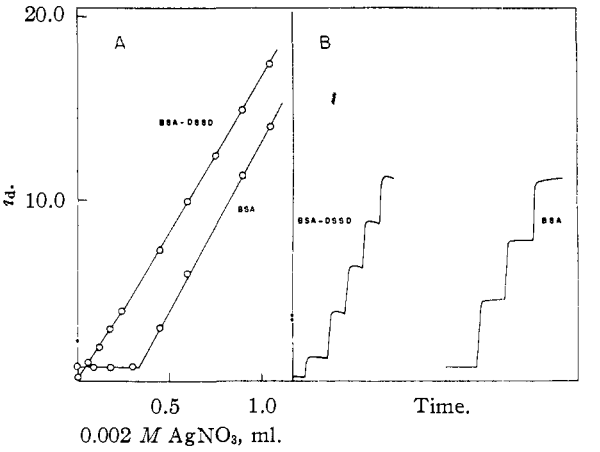


Fig. 5.—Amperometric silver titration curves for bovine serum albumin (1.04×10^{-6} mole) and for bovine serum albumin–dye complex: A, current *vs.* ml. titrant; BSA alone takes up 0.33 ml. Ag^+, the protein–dye complex none. B, a typical set of current *vs.* time curves for different quantities of added titrant; BSA alone reaches and maintains a plateau after each addition, the complex shows a downward drift of plateau current corresponding to a slow removal of free Ag^+ from solution.

Denaturants such as urea or sodium dodecyl sulfate markedly increase the rate of interaction of β-lactoglobulin with DSSD. In an 8 *M* urea solution a sharp end-point is reached within two minutes and does not change significantly for days thereafter. At this end-point 1.08 moles of DSSD had reacted per mole of protein.

It is particularly striking that, under conditions in which the β-lactoglobulin–DSSD reaction is rapid and reaches a sharp end-point, the moles of DSSD reacted corresponds closely to one-half the number of –SH groups in β-lactoglobulin.[29] Equally significant must be the fact that even in urea, which presumably denatures the protein, the disulfide interchange does not continue onward after the original –SH groups of the protein have reacted with the DSSD. In terms of the chain mechanism of Huggins, Tapley and Jensen,[1] one would expect that the new sulfhydryl groups produced in the protein ought to continue to react with DSSD so that the net moles of reacted dye should correspond approximately to the total number of disulfide groups in the protein.

Since the stoichiometry of the reaction with β-lactoglobulin and the behavior in the ultracentrifuge correspond to the observations with serum albumin, it seems reasonable to conclude that the

wise gave high titers, 1.2 moles SH per mole protein, and thus also indicate hydrolysis of S–S bonds.

(27) Over such a long period, the dye itself undergoes some hydrolysis. Offhand one might make a correction for this loss with control tubes containing dye but no protein. However, it is doubtful that this correction is appropriate, since DSSD, being an anion, may form anion complexes with the protein, and its rate of hydrolysis may be modified.

(281 To test the possibility that free radicals are intermediates in the reaction, the exchange with serum albumin was studied in the presence of a variety of free radical trappers (styrene, catechol, etc.). None of these substances slowed down the rate of reaction or changed the stoichiometry (Table 1).

(29) In β-lactoglobulin, two sulfhydryls have been found with an azomercurial,[21] as well as in the amperometric silver titration mentioned in this paper. With ovalbumin, two sulfhydryls seem to be much more accessible than the remaining two or three which occur in this protein.[21,22]

mechanism of the reaction is also similar; that is, that the ideas of equations 8 and 9 are applicable to β-lactoglobulin too.

With ovalbumin, however, some differences seem to be present.[29] It too undergoes a slow reaction with DSSD in aqueous buffer. In 24 hours a photometric titration showed approximately two moles of dye had been cleaved but the reaction had not reached completion.[29] There was also some protein precipitation during the reaction. Essentially similar results were obtained in the absence of air as in its presence. As with β-lactoglobulin, one can attribute the slowness of the ovalbumin reaction to the masked nature of the sulfhydryl group,[22] for again amperometric Ag^+ titrations show the lag characteristic of such a situation. The observation of precipitation may be indicative of some cross linking; *i.e.*, reaction 3 may occur to some extent with ovalbumin.

Photometric titrations also were carried out in the presence of sodium dodecyl sulfate as denaturant. The reaction of ovalbumin and dye was very rapid in solutions of 1 or 3% dodecyl sulfate. At the lower concentration of denaturant a sharp end-point, corresponding to 2 moles of reacted DSSD, was reached in two minutes; a small increase in titer was obtained over an additional 24-hour period. With the more concentrated detergent solution, an end-point at 2 moles was again reached in 2–3 minutes but the reaction continued at an appreciable rate for more than an hour. The more concentrated detergent must either unmask additional mercaptan groups,[29] or perhaps catalyze the hydrolysis of some protein disulfide bonds.[26] It is of interest that similar behavior for detergents has been reported in fibrous proteins[30]; boiling with 10% Duponol markedly increases the –SH titer.

Thus in contrast to the other two proteins, ovalbumin gave no definitive end-point, although the rate of the reaction indicated that two moles of DSSD were reduced more rapidly than succeeding amounts. Likewise, in contrast to serum albumin and β-lactoglobulin, ovalbumin has few if any disulfide linkages.[31] It seems likely, therefore, that the mechanism of equations 8 and 9 is not operative with this protein. The rapid removal of two moles of DSSD and the observation that two of the –SH groups of ovalbumin are much more accessible than the others[21] fit the mechanism of equation 2. It is these two mercaptans which probably are reacting first on a mole per mole basis with DSSD, and it is the others which continue the succeeding slow reaction. It is pertinent to mention in this connection that in dialysis experiments with DSSD and ovalbumin, in contrast to the other proteins, a diffusible yellow component with absorption peak at 490 mμ appeared outside the protein-containing bag; on the basis of equation 2 free DSH with a peak at 490 mμ should appear.

Some Concentration Effects.—The end-point of the reaction of 1% serum albumin with DSSD was not significantly different from that with 0.1% serum albumin (Table I). Neither was there a detectable change in rate.

On the other hand, the rate, though not the stoichiometry, was sensitive to ionic strength. Thus at *p*H 5 in the absence of any salt or buffer ions, the reaction of DSSD with bovine albumin required about an hour to reach the end-point of 0.35 mole DSSD per mole protein (Table I), whereas the reaction goes to completion in two minutes or less in the presence of buffer or sodium chloride. Similar delays were observed at *p*H 6 in the absence of salts. Since the effect of salt is evident at the isoionic *p*H as well as at *p*H 6 where the protein carries a negative charge, it is unlikely that the increase in rate is merely a non-specific ionic strength effect on reactants of similar charge.

Effect of *p*H.—Most measurements with serum albumin were carried out in an acetate buffer of *p*H 6. However, no large change in stoichiometry (Table I) or rate of reaction was obtained over the *p*H range of 2.5–9.2. In buffers, the reaction was completed in every case within 2 minutes. A slightly higher stoichiometric ratio (Table I) was observed at *p*H 2.5. It is possible that the slow release of a ketone-like substance[32] from acid solutions of serum albumin is due to the cleavage of a thioketal-type linkage with concomitant exposure of a thiol group.[33] During the time required for our experiments, however, the amount of this reaction must have been small.

Conclusions

In reviewing these results with proteins one sees that the reactions of DSSD with serum albumin and with β-lactoglobulin are fundamentally distinct from those of small-molecule thiols, whereas the behavior of ovalbumin is more nearly similar. With the latter protein (which has probably only one disulfide group) free DSH seems to be liberated in the reaction of P–SH with DSSD, the appearance of insoluble protein is evidence for formation of P–S–S–P cross-linkages, and the stoichiometry of the interchange reaction is not sharp. All of these observations fit the pattern of equations 4 and 5, which also describe the behavior of small molecules.

In contrast, with serum albumin and with β-lactoglobulin, *only* the mixed disulfide forms, and there is no evidence for even the slightest amount of reaction 5, *i.e.*, no P–S–S–P is detectable. The special stability of the mixed disulfide, P–S–S–D, probably arises from the stabilizing interactions of the organic residue, D, with the protein. In any event, to account for the stoichiometry, equations 4 and 5 (or their analogs for protein, (2) (3)) must be abandoned and replaced by equations 8 and 9.

In outlining a mechanism of greater molecular detail, we encounter no problem with equation 8 since it is comparable to (4) for simple molecules. In contrast, equation 9 cannot be a simple direct reduction of the S–S group of the protein by DSH, even though this is the net result stoichiometrically. The original thiol group of the protein must be in-

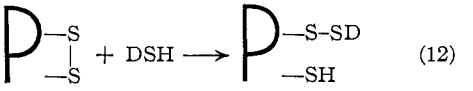
(30) E. J. Van Scott and P. Flesch, *Science*, **119**, 70 (1954).

(31) G. R. Tristram in "The Proteins," edited by H. Neurath and K. Bailey, Vol. I, Academic Press, Inc., New York, 1953, p. 217.

(32) P. Bro and J. M. Sturtevant, Abstracts of the 132nd National Meeting of the American Chemical Society, New York, Sept. 8–13, 1957, page 16-I.

(33) An attempt was made to produce a thioacetal with the mercaptan of serum albumin by adding propionaldehyde to the solution. No significant difference in stoichiometry of the reaction with D-S-S-D was observed (Table I), however.

volved in this reduction reaction. A striking justification for this statement can be found by considering in detail certain aspects of the behavior of serum albumin. One mole of this protein contains 0.67 mole of protein molecules with –SH, 0.33 without. When 0.34 mole of DSSD in reaction 8 has produced 0.34 mole of DSH, one might reasonably expect the dye thiol to react with one of the many S–S groups of *non*-mercapto serum albumin, as well as with the S–S of mercapto serum albumin (with perhaps a statistical advantage, due to concentration, of a factor of 2 for the latter). In any event if the reaction were directly of the type

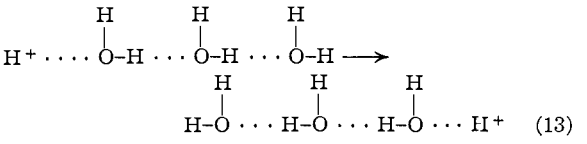


(12)

we should still be left with 0.33 mole of unreacted mercapto serum albumin, and there is no obvious reason why the latter should not cleave 0.33 additional mole of DSSD. Actually the reaction stops abruptly when a *total* of only 0.34 mole of DSSD has been cleaved.

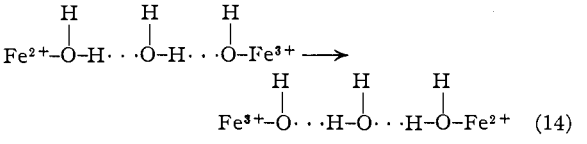
It seems, therefore, that the DSH produced in (8) in some way seeks out the protein molecules which still have their original thiol groups and reacts only with *their* S–S groups and in such a way that the thiol group is simultaneously removed. In other words, the presence of an –SH on the protein must greatly facilitate the reaction of one of its S–S groups with the DSH molecule. With serum albumin there are some grounds for thinking that this might be a neighboring-group effect, *i.e.*, an S–S and S–H being in juxtaposition,[20] but it seems improbable that such would also be the case for both thiol groups of β-lactoglobulin.

We are tempted to ask, therefore, whether the reduction might not be possible over a relatively large distance, perhaps by a process analogous to the Grotthus mechanism. Conventionally, the latter refers to the transfer of an H^+ by means of a chain of oriented water molecules



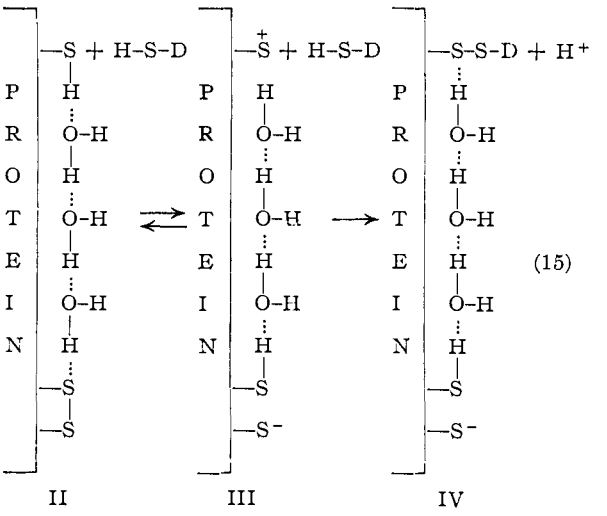
(13)

It has been suggested also that an electron may be transferred[34,35] by means of an H atom over a water bridge between two atoms of different state of oxidation



(14)

It seems obvious that one may extend these concepts and also write an analogous equation for the transfer of a hydride ion, H^-, between groups of different oxidation state separated in space but connected by a bridge of oriented water molecules, *e.g.*



(15)

Thus one can visualize a pair of electrons from a protein –SH group being transferred to the oxidizing S–S moiety at some distance away, as shown in (III). Under normal circumstances, structure III may be present to only a very minute extent. However, with DSH in the solution the second part of reaction 15 would occur immediately. Hence one would obtain a mixed disulfide between DSH and one of the original thiol groups of the protein, and two new –SH groups would be generated in the protein.

This picture gives a reasonable molecular account of the observed stoichiometry, which shows that $^1/_2$DSSD can remove 1 –SH of the protein. It also affords an explanation of the observation emphasized earlier that the thiol groups produced in the interchange reaction are masked, even though those originally present reacted rapidly with Ag^+ or DSSD. If the S–S group were initially in a masked state, due either to steric arrangements of protein side chains, or to the lattice structure of the hydration water of the protein,[36] it could remain so, for the receipt of a pair of electrons is through the water lattice and not by direct collision with a DSH molecule which has broken through the protecting barrier.

This molecular picture has more general implications beyond being just an explanation of the behavior of certain proteins with disulfide compounds. Although we have described the thiol–disulfide reaction in terms of its interchange aspects, it is clearly also an oxidation and reduction, the original thiol of the protein being oxidized, an S–S of the protein being reduced. If, as seems likely from the discussion above, this oxidation–reduction can occur between widely separated –SH and S–S groups, through the medium of H^- transport in the water lattice, it seems reasonable that reducing substrates other than DSH should be able to function in a similar manner. Such a mechanism may be of importance, therefore, in enzyme-catalyzed oxidation–reduction reactions. The mechanism of "energy-transport" between substrates or between

(34) R. W. Dodson, *J. Phys. Chem.*, **56**, 852 (1952); W. L. Reynolds and R. W. Lumry, *J. Chem. Phys.*, **23**, 2460 (1955); J. Hudis and R. W. Dodson, THIS JOURNAL, **78**, 911 (1956).

(35) C. Reid, "Excited States in Chemistry and Biology," Academic Press, Inc., New York, N. Y., 1957, p. 115.

(36) I. M. Klotz and J. Ayers, THIS JOURNAL, **79**, 4078 (1957).

coenzymes and substrates, or between different enzymes in multiple-enzyme systems, particularly those in a fixed matrix such as in mitochondria or in chloroplasts, has been the subject of much serious consideration.[37-40] In view of the observations described in this paper, it seems reasonable that the transport of electrons could be effected generally by the movement of hydride ions across water bridges from –SH to S–S groups, similar to the picture visualized in equation 15. The mercaptan and disulfide could be on the same protein or could be on adjacent enzyme molecules in a linked enzyme system. Thus a reducing substrate acting on one protein could have its electrons transferred to an oxidizing molecule at a substantial distance away on the same enzyme or even on another enzyme linked to the first through a water bridge.

A particularly interesting feature of this scheme is that it provides a mechanism for either a one-electron or two-electron transfer, the latter being effected by transfer of an H^- ion, the former by an H· radical,[41] in each case from –SH to S–S through interconnecting water bridges.

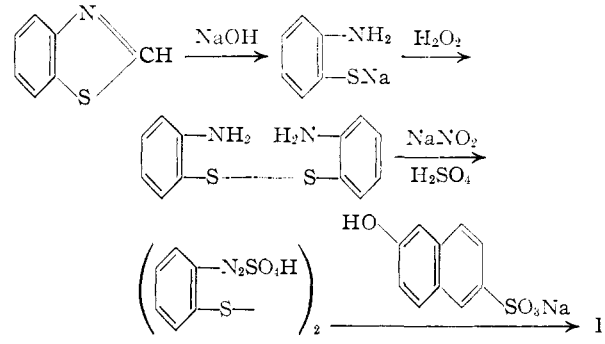
The mechanisms visualized above also provide a framework for the interpretation of many light-activated phenomena in protein behavior which seem to involve energy transfer over long distances. For example, in the myoglobin Fe–CO complex, the action spectrum for the release of CO indicates that electromagnetic energy absorbed by amino acid residues in the protein can affect the spatially-removed heme group.[42] If the absorbed radiation produces an –S· free radical, either directly by splitting an S–S bond symmetrically, or indirectly by perhaps releasing an H· radical, from tyrosine or aromatic rings, which then reacts with S–S, one can readily visualize the oxidation of the heme iron taking place through the transfer of an electron, by means of H· transport through the water, to a distant –S· free radical to form the more stable –SH. It is important to keep in mind in this connection that S–S linkages, as well as aromatic amino acid residues of proteins, absorb ultraviolet light in the region of 250 mμ[43] and that such light can dissociate disulfides into –S· free radicals.[44-46]

It is also apparent that the concept of transport of electrons by H^- ion transfer through the lattice of water molecules as visualized in equation 15 could provide a basis for the interpretation of long-range and coöperative energy-transfer processes such as are involved in photosynthesis or in other light-activated oxidation–reduction phenomena. It is of interest in this connection that mercaptans seem to be implicated in the photosynthetic mechanism[43] and in the photoreception process.[47]

Thus the transport mechanism visualized by equation 15 provides a molecular basis not only for the understanding of the relatively simple problem of thiol–disulfide interchanges but also for the interpretation of a variety of phenomena characteristic of protein molecules or of organized protein-containing systems.

Experimental

Synthesis of Disodium Salt of 2,2′-(2-Hydroxy-6-sulfonaphthyl-1-azo)-diphenyl Disulfide.—This dye was prepared by the following sequence of reactions



One hundred ml. of benzothiazole was boiled with 100 ml. of 50% sodium hydroxide under reflux for three hours.[48] Unreacted benzothiazole was removed by steam distillation. Half of the remaining solution of sodium *o*-aminothiophenol was oxidized at 0° by addition of 42 ml. of 30% hydrogen peroxide, dropwise and with stirring. The crude 2,2′-diaminodiphenyl disulfide was filtered off and dissolved in hot 95% ethanol. To prevent oxidation and discoloration of the disulfide, 50 ml. of a 2–4% aqueous solution of sodium bisulfite was added to the hot filtrate. The solution was then cooled and to it was added with stirring, 80 ml. of water. The crystalline yellow 2,2′-diaminodiphenyl disulfide obtained melted at 91–93°.

Tetrazotization and coupling was carried out along the general lines used by Burawoy and Turner.[49] Nitrosyl sulfuric acid was prepared by addition of 3.6 g. of sodium nitrite to 25 ml. of concentrated sulfuric acid at 0°. Five grams of diaminodiphenyl disulfide was dissolved in 17 ml. of concentrated sulfuric acid at room temperature. The latter solution was then cooled to and maintained at 0–5° and the nitrosyl sulfuric acid added to it. A separate solution was then prepared containing 3 g. of urea, 160 g. of sodium bicarbonate and 10 g. of previously recrystallized sodium 2-naphthol-6-sulfonate, all dissolved in 700 ml. of water. After cracked ice had been added to the latter solution, the tetrazonium salt solution was added dropwise and with stirring, the mixture being maintained at 0–5°. Stirring was continued for one hour after the last addition of tetrazonium salt. The *p*H of the solution was then adjusted to 6 with 12% sulfuric acid, and the impure dye was separated by filtration. The solid was washed with a few ml. of 3–4% sulfuric acid, then with ice-water. After being dried *in vacuo*, the dye was washed with a few ml. of benzene and thereafter with a little ether. Subsequent drying gave an impure product with a molecular extinction coefficient of 25,000 at 515 mμ. The impure material was recrystallized several times by dissolving it in 1:1 aqueous ethanol, adjusting the *p*H to about 6 and adding acetone dropwise. The recrystallized material was then dried over phosphorus pentoxide *in vacuo*. Recrystallization was terminated when a constant value, 45,000, was reached for the molecular extinction coefficient.

(37) A. Szent-Gyorgyi, *Science*, **93**, 609 (1941).
(38) K. Wirtz, *Z. Naturforsch.*, **2a**, 267 (1947).
(39) T. A. Geissman, *Quart. Rev. Biol.*, **24**, 309 (1949).
(40) T. Bücher, *Advances in Enzymology*, **14**, 1 (1953).
(41) The movement of H· radicals in water is analogous to that previously postulated by Dodson[34] and Reynolds and Lumry[34] to account for the kinetics of oxidation of ferrous ion, and some related reactions, in aqueous solution.
(42) T. Bücher and J. Kaspers, *Biochim. Biophys. Acta*, **1**, 21 (1947).
(43) J. A. Barltrop, P. M. Hayes and M. Calvin, THIS JOURNAL, **76**, 4348 (1954).
(44) S. F. Birch, T. V. Cullum and R. A. Dean, *J. Inst. Petroleum*, **39**, 206 (1953).
(45) G. H. Crawshaw and J. B. Speakman, *J. Soc. Dyers Colourists*, **70**, 81 (1954).
(46) K. E. Russell and A. V. Tobolsky, THIS JOURNAL, **76**, 395 (1954).
(47) G. Wald and P. K. Brown, *J. Gen. Physiol.*, **35**, 797 (1951–1952).
(48) J. A. Gardner and Monsanto Chemicals Ltd., British Patent 558,887 (Jan. 26, 1944).
(49) A. Burawoy and C. Turner, *J. Chem. Soc.*, 469 (1950).

Anal. Calcd. for $C_{32}H_{20}N_4O_8S_4Na_2$: S, 16.8; N, 7.35. Found: S, 16.2, 15.8; N, 6.87, 7.27.

Traces of inorganic salt probably still were present.

Reagents.—The proteins bovine serum albumin and β-lactoglobulin were crystalline samples purchased from Armour and Co. Crystalline ovalbumin was obtained from Worthington Biochemical Corporation. Iodinated bovine albumin was the same as used previously.[21]

Reagent grade urea was further cleaned of traces of metals by the procedure recommended by Benesch, Lardy and Benesch.[22] Other organic chemicals were high purity commercial grades and were used directly. Inorganic substances, as well as the citrate and acetate salts and acids used for buffers were reagent grade materials.

Oxygen-free solutions were obtained by bubbling through specially pre-purified nitrogen (Matheson Co.) which was warranted to contain less than 0.001% O_2.

Osmotic Pressure Measurements.—Molecular weights were determined with the Bull osmometer.[50] To test the reliability of our technique measurements were made first with bovine albumin alone, in 0.2 *M* acetate buffer, *p*H 6.0. The average of twelve determinations in the concentration range of 0.5–1.5% was 66,300 ± 4100. The dye–protein complex, in the same buffer, did not give constant readings within a 24-hour period. Molecular weights rose in the equilibration interval between 17 to 40 hours, tending toward values above 60,000. Examination of the buffer solution outside the protein bag showed a slight yellow color. It seems likely that traces of xanthate or other sulfur compounds remaining in the cellophane bag are leached out during equilibration; these break some of the P-S-S-D linkages and release the dye (probably as a xanthate derivative) from the protein.[51] If the freed dye diffuses out only slowly, the molecular weight from osmotic pressures would tend to be low initially and to rise with time. The values obtained nevertheless indicate that the protein–dye complex is a monomer, since the dimer would have a molecular weight near 130,000. However, in view of the uncertainty of these results, sedimentation experiments also were carried out.

Ultracentrifuge Measurements.—Molecular weights of the bovine albumin–dye complex were determined by the approach-to-equilibrium method of Archibald,[52] as modified by Klainer and Kegeles,[53] and by Ginsburg, Appel and Schachman.[54] Solutions of dye and protein in acetate buffer at *p*H 6 were mixed to give a final concentration of protein of 1.43×10^{-4} *M* (1%) with all the initial sulfhydryl groups combined with dye. The solutions were then centrifuged at the low speed of 6,387 r.p.m. and photographs were taken over a period of four hours. As recommended by Schachman[54] we placed approximately 0.1 cc. of Dow-Corning No. 555 silicone at the bottom of the cell, and 0.6 cc. of the protein solution above it so that the gradient at the bottom of the cell could also be used for computation. The concentrations of protein in the solutions were determined in arbitrary units by a separate sedimentation in a synthetic boundary cell.[53] The angle of the schlieren diaphragm was maintained between 60 and 80°.

The photographic plates were placed in an enlarger and the images, enlarged about 13 times, traced on graph paper. The appropriate distances were then read off and the summations and computations made in the manner recommended by Schachman.[54] The molecular weights computed from measurements of the top of the cell were consistently higher than those obtained from the bottom, in contrast to what one would expect if a heavier component than the original protein were present. This error probably arises from the bias of the experimenter in setting the exact height of the ordinate intercept, the gradient of the concentration. In any event, all of the computed molecular weights were within 8% of the average value 74,100. This result clearly indicates that dimers of serum albumin were not formed in the reaction with disulfide dye.

After the photographs required for concentration determinations were taken in the synthetic boundary cell, the ultracentrifuge was speeded up to 60,000 r.p.m. to sediment the protein more rapidly. The color of the dye was observed to move along with the protein.

Conventional sedimentation velocity experiments were also carried out. The schlieren photographs of protein–dye complex looked identical with those of protein alone (Fig. 4). Sedimentation coefficients also were computed and did not differ in the presence of dye. These experiments were carried out with β-lactoglobulin as well as with bovine serum albumin. With both proteins also, the boundary of the dye color essentially coincided with the position of the schlieren peak.

Dialysis Experiments.—The extent of binding was measured by the dialysis technique described previously[13]; 10 ml. of 0.2% protein inside a cellophane bag was equilibrated with 10 ml. of a dye solution outside the bag. Separate tubes without protein were used to correct for the uptake of dye by the cellophane. The corrections in these experiments were very large, probably because of a chemical reaction between traces of xanthates (or other sulfur compounds) in the bags and the added disulfide dye. Equilibration in each tube was effected by mechanical shaking for 24 hours.

Amperometric Titrations.—The apparatus, conditions and procedure recommended by Benesch, Lardy and Benesch[22] were used in all measurements.

Acknowledgment.—This investigation was assisted by a grant from the National Science Foundation, and in its early phases by a research grant (RG-4134) from the National Institutes of Health, United States Public Health Service.

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(50) H. B. Bull, *J. Biol. Chem.*, **137**, 143 (1941). We are indebted to Dr. H. A. Fiess for carrying out these measurements.

(51) Some measurements also were made with the albumin-dye complex to which a mole of Hg^{++} was added per mole of protein. A molecular weight of 63,800 ± 2800 was obtained as the average of four determinations. In this case too there was evidence of some mercury-dye complex in the solution outside the protein bag.

(52) W. J. Archibald, *J. Phys. Colloid Chem.*, **51**, 1204 (1947).

(53) S. M. Klainer and G. Kegeles, *Arch. Biochem. Biophys.*, **63**, 247 (1956).

(54) A. Ginsburg, P. Appel and H. K. Schachman, *ibid.*, **65**, 545 (1956). We are indebted to Dr. Schachman for his advice and assistance during these experiments.