I. 3. DISULFIDE BONDS IN PROTEINS *

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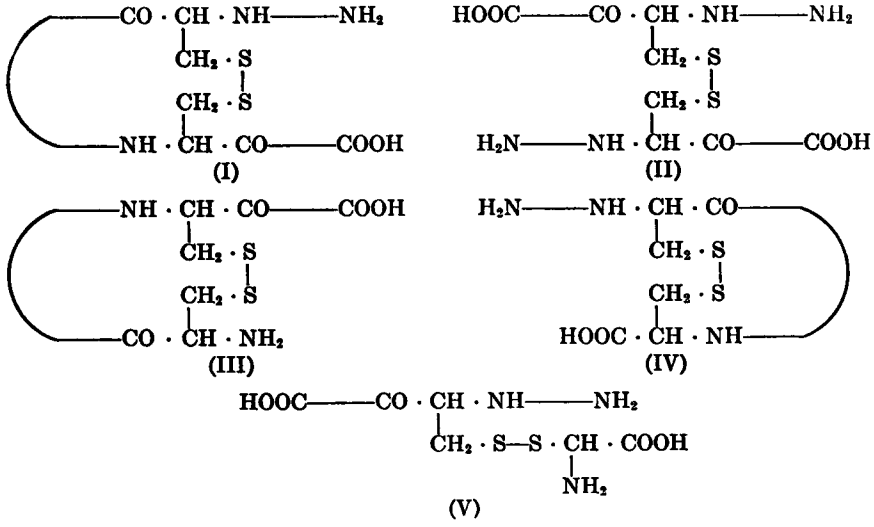
I. The Linkage of Cystine in Proteins

Cystine is unique among the natural amino acids in that it contains two amino groups and two carboxyl groups. It can, therefore, give rise to different types of branching some of which are shown below in the diagrams (I–V).



In order to differentiate between structures of the Types (I) and (II) we have attempted to determine the molecular weight of proteins before and after cleavage of the disulfide bonds. A molecule possessing structure (I) would not change in weight since cleavage of the intrachain bond does

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not involve fragmentation of the molecule. Molecules of the Type (II), however, would be split into subunits of a lower molecular weight if the —S—S— bond is split. The reagents used for the cleavage of the disulfide bonds were thioglycolate, sulfite and performic acid. Molecular weights were determined by the light scattering method (*1*).

Bovine γ-globulin, pepsin, edestin, and ovalbumin were found to have the same molecular weights before and after cleavage of the disulfide bonds with performic acid. This suggests that these molecules possess only intrachain disulfide bonds as shown in Diagram (I). Bovine γ-globulin and edestin gave the same results with sulfite or thioglycolate. The turbidity of ovalbumin solutions in sulfite and thioglycolate increased with time; evidently, cleavage of the disulfide bonds of ovalbumin is followed by formation of new intermolecular disulfide bonds and subsequent aggregation. A similar result was obtained when bovine serum albumin was treated with thioglycolate. The molecular weight of bovine serum albumin remained unchanged in sulfite, but decreased after exposure to performic acid (*2, 3*). Determination of the terminal α-amino groups seems to indicate the cleavage of peptide bonds of serum albumin by the oxidizing agent. The cleavage is, therefore, reconcilable with a single peptide chain as the backbone of the serum albumin molecule [Type (1)] although interchain bonds of the Type (II) cannot be excluded. It has been shown by the work of other laboratories that intrachain bonds of Type (I) are also present in ribonuclease (*4*), whereas interchain bonds of Type (II) are found in the insulin molecule (*5*).

Structures of Type (III) were first discovered in oxytocin and vasopressin (*6*), later in chymotrypsin (*7*). We analyzed 18 different proteins, for the presence of an N-terminal group in cystine. The direct treatment of the proteins with dinitrofluorobenzene with Sanger's method (*8*) leads to the formation of DNP-cystine which, however, is destroyed by acid hydrolysis. We oxidized, therefore, the dinitrophenylproteins, before hydrolysis, with performic acid (*7*). DNP-cysteic acid, which is stable to acid hydrolysis, was found only in the hydrolyzates of chymotrypsin and horse serum albumin, but in none of the following proteins: bovine serum albumin and γ-globulin, fibrinogen, trypsin, casein, ovovitellin, lactalbumin, β-lactoglobulin, ovalbumin, pepsin, rennin, dog ferritin, phaseolin, legumin, glycinin, and edestin. The occurrence of structure (III) in equine serum albumin had been reported earlier by Titani *et al.* (*9*).

In order to detect structures of Type (IV) proteins were exposed to hydrazinolysis (*10*). In view of the lability of cystine in this procedure, the proteins were pretreated with performic acid. No free cysteic acid was found in any of the hydrazinolyzates. Hence, none of the proteins seem to contain the structure shown in (IV). Likewise, structure (V) does not

seem to occur in proteins. A search for this structure was made by oxidation of some of the proteins with performic acid, precipitation of the oxidized proteins with 5 *M* sodium chloride solution, and examination of the supernatant solution for cysteic acid (*11*).

The principal result of the analyses described in the preceding paragraphs is that most of the disulfide bonds in proteins occur as intrachain bonds [Type (I)], that only rarely interchain bonds [Type (II)] or cystine residues with a free amino group [Type (III)] are encountered, and that none of the Types (IV) and (V) seem to occur.

II. Optical Rotation Studies

In the last few years, the decrease in levorotation of proteins after treatment with performic acid (*12*) or mercaptoethylamine (*13*) has been attributed to changes in the conformation of the peptide chains. It seemed to us that the enormous difference between the rotatory power of cystine and cysteine should be taken into consideration in the interpretation of these results. Therefore, several proteins were oxidized with performic acid (*3*), precipitated with 5 *M* NaCl solution, dialyzed to remove salts, and lyophilized. The rotation of both the native and the oxidized protein was determined in 88% formic acid. The specific rotations of cystine and cysteic acid in the same solvent were found to be −285° and +5.5°, respectively. From the cystine content of the proteins we have calculated the change in rotation which can be attributed to the conversion of cystine into cysteic acid. It can be seen from Table I that this change, shown in

TABLE I*

Optical Rotation of Native and Oxidized Proteins

	Δ(Natural → Oxidized) A (Observed)	Δ(SS → $2SO_3H$) B (Calculated)	Δ(*x*) C (Calculated)
Bovine Serum Albumin	+22.5° ± 1.2(+28°)[a]	+17.3° (+22°)	+5.2° ± 1.2(+6)
Edestin	+6.4° ± 0.6	+2.8°	+3.6° ± 0.6
Ovalbumin	+4.9° ± 1.0	+1.5°	+3.4° ± 1.0
γ-Globulin	+0.9° ± 0.7(+3°)[a]	+7.0° (+9°)	−6.1° ± 0.7(−6)
Pepsin	−1.0° ± 0.6	+6.8°	−5.8° ± 0.6
Insulin	(+38°)[b]	(+35°)[c]	(+3°)

* Experimental data are given in a communication by Turner *et al.* (*15*). The statement made in this communication (*15*) that performic acid is reduced by ethanol is erroneous.

[a] Values of Markus and Karush (*13*). The value of B was calculated from the specific rotation of cystine in aqueous solution, pH 7 (*14*).

[b] In dimethylformamide (*12*).

[c] Calculated from the specific rotation of cystine in formic acid.

the table as $\Delta(SS \rightarrow 2\,SO_3H)$ is in some instances of the same order as the observed total change of specific rotation, Δ(Natural → Oxidized). These values agree well with those observed by other authors who used oxidizing (*12*) or reducing (*13*) agents to cleave the disulfide bonds. Since oxidative cleavage leads essentially to the same results as reductive cleavage, the change in $[\alpha]_D$ must be attributed primarily to the cleavage of the disulfide bonds and not to other phenomena which might accompany this reaction.

If we deduct the calculated value of $\Delta(SS \rightarrow 2\,SO_3H)$ from the observed total change in specific rotation, Δ(Natural → Oxidized), we obtain the $\Delta(x)$ values shown in the last column of the table. They may, tentatively, be considered as reflecting configurational changes which result, secondarily, from the cleavage of the disulfide bonds. The positive sign of the $\Delta(x)$ values of bovine serum albumin, ovalbumin, edestin, and insulin might indicate that the cleavage of the disulfide bonds is accompanied by an increase in folding or in helical structures. The negative sign of the $\Delta(x)$ values of bovine γ-globulin and pepsin might reflect unfolding of the peptide chains or loss of helical character after cleavage of the disulfide bonds in these molecules. In considering the results obtained with pepsin it must be pointed out that pepsin is stable at low pH values and may not be denatured under the conditions of performic acid oxidations.

The enormous levorotation of cystine, which is quite different from the weak dextrorotation of cysteine or cysteic acid, is also found in oxidized glutathione but not in homocystine (*14*). Fieser (*14*) attributes the difference between cystine and homocystine to the formation of hydrogen bonds between the NH and CO groups in the α-position of cystine or its peptides. In homocystine, hydrogen bonding is prevented by the additional CH_2 group. We are grateful to Dr. E. Campaigne, of this department, who demonstrated to us by means of a molecular model of cystine that the angles in the C—S—S—C chain force the molecule to assume a twisted configuration, shown schematically in Fig. 1. In this structure the asym-

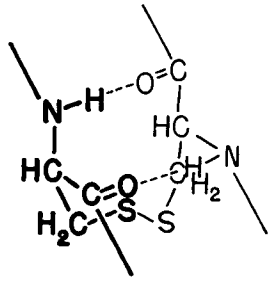

FIG. 1. Conformation of cystine.

metric carbon atoms with the adjacent atoms cannot rotate around the S—C bond, and are fixed in a definite orientation. This may be responsible for the high specific rotation of cystine and also for the increase in dextrorotation when proteins which are rich in disulfide bonds are treated with reagents which cleave these bonds. We believe that it is not necessary to invoke an increase in helical structure as an explanation for the large increase in dextrorotation in proteins of this type.

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Discussion

BENESCH: I would like to mention that Dr. Gerald Oster, who was invited to this symposium but could, unfortunately, not attend, has written a review on disulfide chemistry in the *J. Soc. Cosmetic Chemists* [**5**, (4), 286, 1954] in which he speculates on the reasons for the enormous rotation of cystine. He reaches the conclusion that cystine has sufficient characteristics of a helix to account for this rotation.

HAUROWITZ: Yes.

BENESCH: The other thing I want to mention is that we recently came across some older work which again points up the uniqueness of cystine. I am referring to the work of R. A. Gortner and W. F. Hoffman (*J. Am. Chem. Soc.* **43**, 2199, 1921) and C. G. L. Wolf and E. K. Rideal (*Biochem. J.* **16**, 548, 1922) who found that dibenzoyl cystine forms gels in concentrations as low as 0.2%. This finding prompted us to investigate the possible gelling properties of a sample of dibenzoyl *homocystine* which we had around. The results were entirely negative, which again shows that there is something very special about the configuration of the cystine disulfide bond.

KAUZMAN: One other point on cystine. There is a rather large temperature coefficient of the optical rotation of cystine—something like a 5% decrease in magnitude per 10° increase in temperature. I do not believe this shows up in the optical rotation

of serum albumin, which is nearly independent of temperature up to 60° and becomes somewhat more levorotatory above 60°.

HAUROWITZ: We did not investigate at different temperatures. We investigated at 25°.

KAUZMAN: I am sure you realize that it is treacherous on theoretical grounds to interpret the optical rotation changes in a peptide using the numbers that you get out of cystine itself. The optical rotatory behavior of peptides is in general quite different from that of the amino acids from which they are made.

SWAN: I would like to suggest an experimental approach that might help in this problem. H. T. Clarke (*J. Biol. Chem.* **97**, 235, 1932) has prepared the sodium and ammonium salts of *S*-sulfocysteine, and these have surprisingly high rotations in water, the sodium salt ($+1.5H_2O$) having $[\alpha]_D^{23°} - 86.8°$ (*C*, 4.73) and the ammonium salt $[\alpha]_D^{23°} - 104.2°$ (*C*, 4.14). By contrast, cysteic acid has a rotation in water of about +8.7°. It would be interesting to compare the rotations of insulin, insulin in the presence of sulfite, the completely separated chains obtained by the air-sulfite or cupric-sulfite processes, and the mixed A and B chains obtained by performic and oxidation.

KOLTHOFF: I would like to add one more example. We have broken the disulfide bonds of serum albumin with sulfite in guanidine hydrochloride and have found that the optical rotation is *unaffected* by the breaking of 11 disulfide bonds per mole. This is in great contrast to the results of Markus and Karush (*J. Am. Chem. Soc.* **79**, 134, 1957) who split the disulfide bonds with β-mercaptoethylamine using sodium decyl sulfate as the denaturing agent and found a decrease from −72° to −44° as a result of the reduction of the disulfide bonds. We have confirmed their experimental observations and have come to the conclusion that the difference must be attributed to the different structure of albumin denatured with guanidine hydrochloride (or urea) on the one hand, and with detergent on the other. It is, therefore, difficult to draw too many conclusions on the basis of splitting of disulfide bonds.

HAUROWITZ: It is difficult to work with disulfide in reducing media. We investigated bovine serum albumin in sulfite and sulfate for comparison. As I say, we did not find any change of rotation. We did not continue these experiments because I thought that this is just a reversible reaction and the protein has probably such a tendency to return to the original —S—S— structure that we cannot bring about any changes unless we denature at the same time. This I did not want to do. I think the only clear-cut reaction is the oxidation with performic acid. As one of the speakers before said, it is the only reaction which is completely irreversible.

KOLTHOFF: The albumin does not react at all with sulfite; that is, the native albumin. In the native state there could not be any effect because there is no chemical reaction.

LOW: In commenting on the change in the levorotation which you attributed to —S—S— going to $—SO_3$, I was not quite sure of the configurational basis on which you were able to make these calculations. How were you able to come out with numbers? Did you assume in one case that you had a given well-defined configuration and in the other case a complete lack of order?

HAUROWITZ: We observed a value of $[\alpha]_D$ and deducted from it a calculated value obtained in the following way. We measured cystine and cysteic acid in 80% formic acid: the difference between these was about 300°. Cysteic acid, as I remember, has +5° specific rotation, and cysteine has +8°. Cystine has −285°. The difference between cystine and cysteic acid is 290°. We added for each molecule of cystine in the

proteins this difference in $[\alpha]_D$. This gave the calculated value. The deviation between observed and calculated values may be due to configurational changes.

EDSALL: Has the rotatory dispersion of cystine been studied?

HAUROWITZ: I don't know. We wanted to measure the rotatory dispersion.

BOYER: One question that is simple but perhaps you can answer it. Does urea affect the rotation of cystine itself? This is relative to Dr. Kolthoff's comment. Do you know if the rotation of cystine has been measured in high urea concentration?

HAUROWITZ: No, we wanted to avoid denaturing agents.

BOYER: This would be relevant to the spatial structure of the cystine. I am reminded of Dr. Benesch's observation on the rates of reactions of certain compounds with nitroprusside in which he was able to show marked effects of urea on the reactivity. I wonder if the urea would affect the rotation of the cystine?

KAUZMANN: I don't know. I was just asking Kolthoff. I never measured that. It is not very soluble and it is a little hard to measure the rotation.