*THIOLATION OF PROTEINS**

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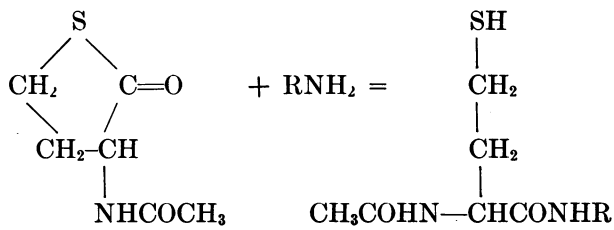
INTRODUCTION

In view of the importance of sulfhydryl groups and disulfide bonds for the biological and mechanical properties of many proteins, we have been interested in finding a method by which these groups could be introduced into proteins *de novo*. We had found previously that *N*-acetylhomocysteine thiolactone (AHTL) reacts smoothly with amino acids to form *N*-acetylhomocysteine peptides:[1]



The application of this reaction to the thiolation of proteins was limited by the fact that a relatively high pH and long reaction times were required to obtain good yields. We have now found that in the presence of silver the thiolation proceeds rapidly at room temperature in aqueous solution at pH 7.5. A similar effect of silver on the aminolysis of certain thiolesters has been reported by Schwyzer.[2] Somewhat unexpectedly, silver and AHTL react to form an insoluble complex. A maximum of 2 Ag^+ per mole of AHTL is bound and one proton is liberated for each equivalent of silver. It is the insoluble silver complex which reacts with protein amino groups.

METHOD OF THIOLATION

The reaction is carried out by treating an aqueous solution containing the protein (3–5 per cent) and AHTL (cf. Table 1) with alternate increments of $AgNO_3$ and NaOH to keep the pH at 7.5, until a total of 1 mole of silver per mole of AHTL has been added. The reaction is complete when all the Ag AHTL complex has dissolved. This takes about 1 hour. The clear yellow solution of the silver mercaptide of the thiolated protein is adjusted to pH 2.5 and enough thiourea is added to convert all of the silver into the soluble $Ag(thiourea)_2^+$ complex. This complex ion is removed with Dowex 50, and the protein is washed off the resin with acidified 1 *M* thiourea solution. The effluent protein solution is then brought to pH 7 and passed through Amberlite IRA 400 in order to remove some N-acetylhomocysteine which is formed as a by-product due to hydrolysis of AHTL. The protein solution is finally freed of thiourea and salts by dialysis under nitrogen and lyophilized.

Some special advantages of this method might be emphasized:

1. The —SH group is linked to the protein by a stable, covalent bond, i.e., a peptide bond.

2. The —S—CO— bond serves the dual purpose of activating the carboxyl group and protecting the —SH group in the thiolating reagent.

3. After the opening of the ring, the —SH group is protected by silver.

4. The reaction proceeds under very mild conditions, i.e., in aqueous solution, at neutral pH and room temperature.

5. The specificity of the method for primary amino groups seems to be of a high order, since a close correspondence was found between loss of amino groups and formation of —SH groups (Table 1).

6. The number of —SH groups introduced can be varied over a wide range simply by changing the ratio of AgAHTL to protein (Table 1).

TABLE 1

THIOLATION OF GELATIN

Moles Reagent per $-NH_2$ Group	Groups per 10^5 Gm. Initial $-NH_2$*	Final $-NH_2$*	$-NH_2$ Blocked	—SH Formed†
1	32.5	22.3	10.2	10.7
2	36.1	19.8	16.3	16.7
3	32.5	12.3	20.2	20.3
4	36.1	11.6	24.5	22.5
10	36.1	6.4	29.7	29.5

Determined by formol titration.

† Determined both according to P. D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331, 1954, and R. Benesch and R. E. Benesch, *Biochem. et. Biophys. Acta*, **23**, 643, 1957.

PROPERTIES OF THIOLATED GELATINS

These gelatin derivatives are remarkably stable in the lyophilized state provided they are kept dry since their —SH titer does not change appreciably over a period of several weeks. They dissolve readily in water to form clear, colorless solutions. They form heat-reversible gels with melting points only somewhat lower than those of the gelatins from which they were derived. As would be expected from the decrease in the number of lysine amino groups, their isoelectric points are lower (Table 2). Modification of the lysine ϵ-amino groups of proteins is known to reduce their susceptibility to tryptic hydrolysis.[3] Table 3 shows this to be the case with thiolated gelatins.

TABLE 2

ISOELECTRIC POINTS OF THIOLATED GELATIN

	I.P.*
Control "A" gelatin	6.56
29 —SH per 10^5 gm.	4.51
Control "B" gelatin	5.06
24 —SH per 10^5 gm.	4.21

* Isoelectric points were determined by the method of Janus *et al.*, *Research*, **4**, 247, 1951.

TABLE 3*

TRYPTIC HYDROLYSIS OF THIOLATED GELATIN

Gelatin	Groups per 10^5 Gm. Initial $-NH_2$	Maximum No. Peptide Bonds Hydrolyzed by Trypsin
Control	35.8	51
Thiolated	8.3	38

* The intramolecular disulfide derivative (cf. below) was used for this experiment in order to avoid any direct effect of the -SH groups on the enzyme.

The built-in —SH groups were examined by means of their characteristic absorption in the ultraviolet. This spectrum, with a maximum at about 238 mμ, is a property of mercaptide anions (RS^-)[4] and can therefore be used to determine the degree of ionization of —SH groups.[5] The effect of pH on the spectra of a gelatin containing 26 —SH groups per 10^5 gm. is shown in Figure 1. When the —SH groups

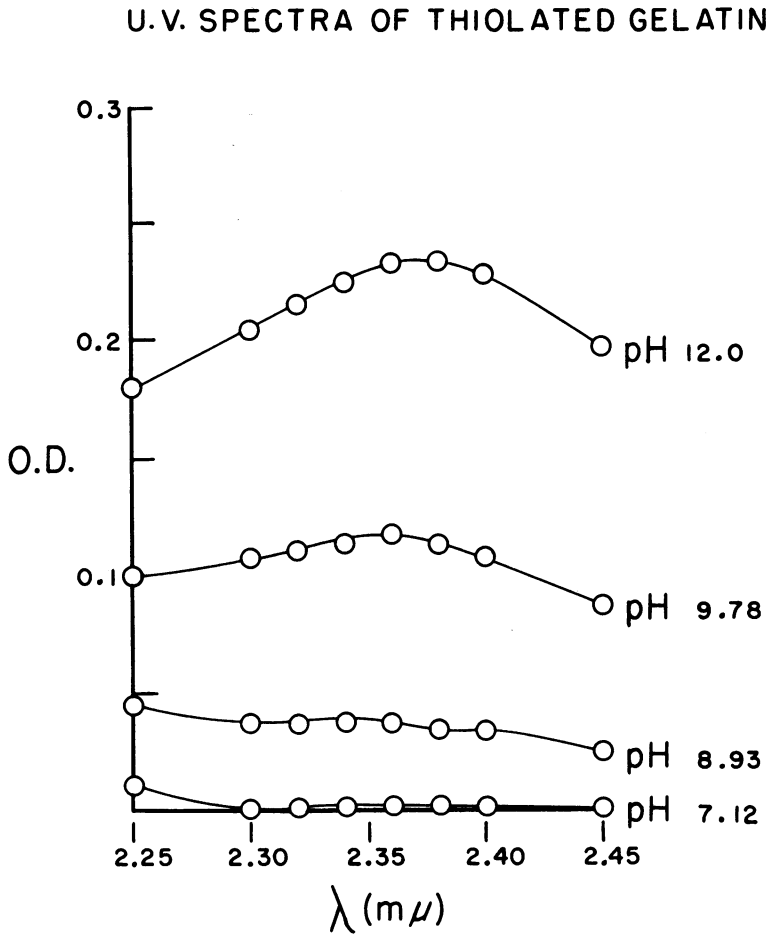


FIG. 1.—These spectra were obtained by methods previously described.[5] Each spectrum represents the difference between the optical density at the given pH and the optical density of the same solution at pH 4.6.

in this gelatin are oxidized to disulfides, spectra identical with that of the —SH form at pH 7.12 are obtained at all pH values up to pH 12. From these data it becomes evident that the built-in —SH groups are half-dissociated at a pH of 9.8. This is in close agreement with the pK of N-acetylhomocysteine itself, determined by the same method (10.0).

DISULFIDE DERIVATIVES OF THIOLATED GELATINS

The —SH groups of the thiolated gelatins can be quantitatively oxidized to disulfide bonds with ferricyanide in neutral solution. The properties of the resulting

disulfide gelatins depend entirely on the concentration of the protein during the oxidation. These reactions are represented schematically in Figure 2.

a) Oxidation in Dilute Solution (Intramolecular Disulfide Gelatin).—A 0.2 per cent solution of thiolated gelatin is treated with a slight excess of 0.1 *M* potassium ferricyanide at pH 7 until the nitroprusside test is negative. Ferricyanide and ferrocyanide ions are removed with Amberlite IRA 400. After dialysis the solution is lyophilized. The product thus obtained is freely soluble in water. Viscosity measurements point to the fact that no intermolecular aggregation has taken place. This is further supported by the results obtained with the ultracentrifuge,[6] which show no significant difference between the average molecular weight of the original gelatin, the same gelatin after the introduction of 30 —SH groups per 10^5 gm., and the disulfide gelatin obtained from this by oxidation as described above.[7]

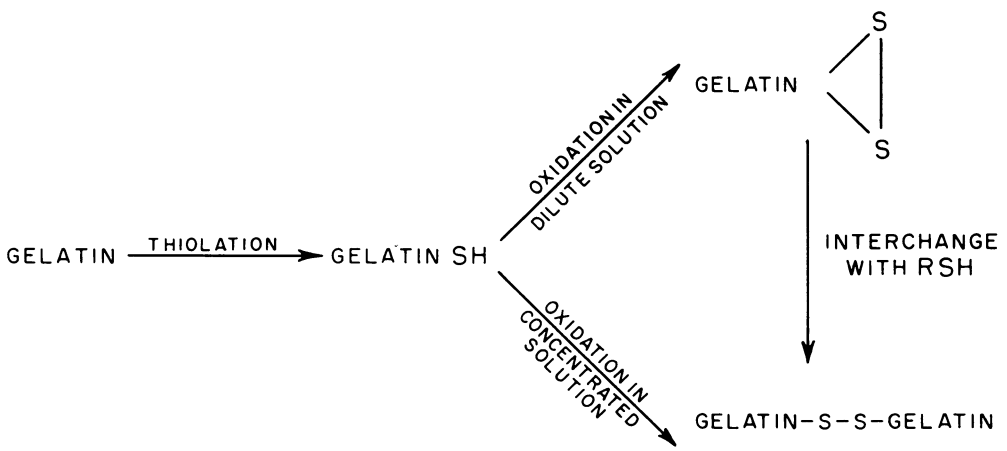


FIG. 2.

These intramolecular disulfide gelatins show a dramatically reduced tendency to form gels. This is illustrated in Figure 3. It is clear that this drop in melting point occurs very sharply at a critical level of disulfide bonds. When the disulfide content is greater than 10 —SS— bonds per 10^5 gm., the protein does not gel at all in concentrations below 4 per cent. These results lead to the conclusion that the intramolecular disulfide bonds in these derivatives interfere with the normal hydrogen-bonding mechanism which is responsible for the gelation of ordinary gelatin.

b) Oxidation in Concentrated Solution (Intermolecular Disulfide Gelatin).—When solutions of thiolated gelatins with 17 or more —SH groups per 10^5 gm. are treated with slightly less than the theoretical amount of ferricyanide at a protein concentration of 5 per cent or more, the solution sets almost instantly to a clear, colorless, rigid gel. These gels do not melt even at 100° C. and are insoluble in concentrated solutions of urea and guanidinium chloride. They can, on the other hand, be "melted" by treatment with an excess of a suitable thiol, such as β-mercaptoethylamine. The gelation mechanism involving hydrogen bonds has evidently been replaced largely, if not entirely, by intermolecular disulfide bridges. It is well known[8, 9] that intramolecular disulfide bonds can be changed to intermolecular disulfide bonds in the presence of catalytic amounts of thiols in alkaline solution

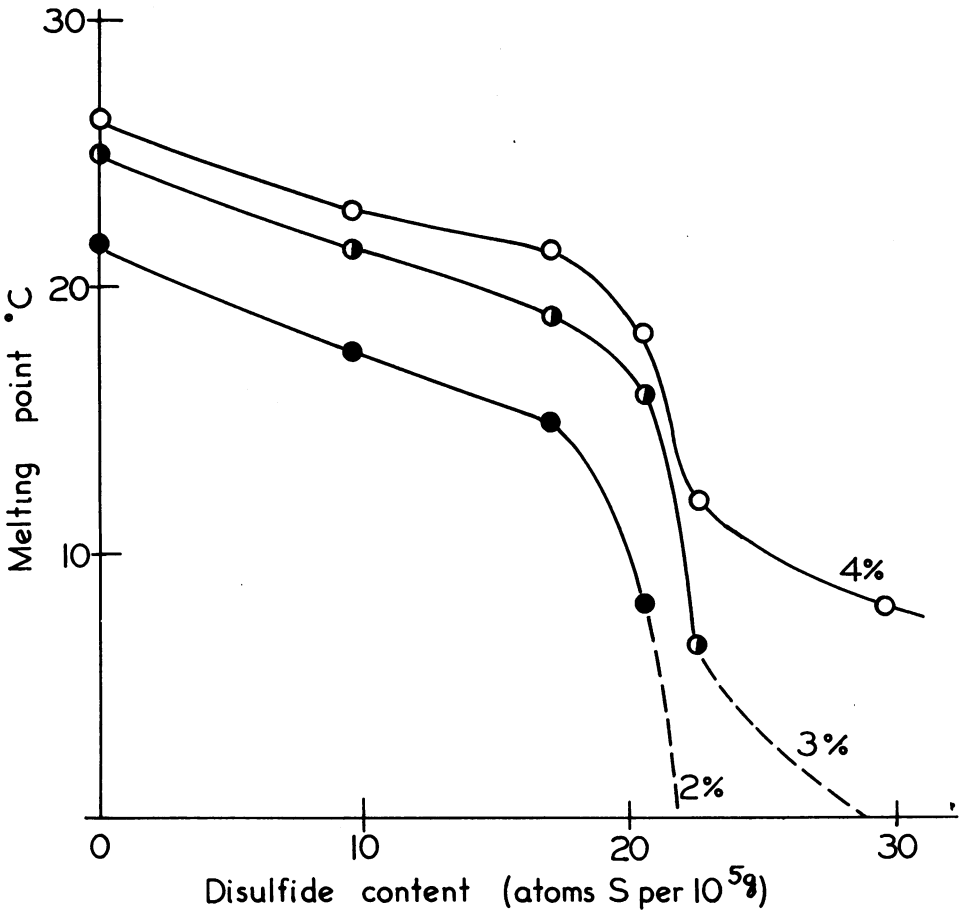


FIG. 3.—Each point represents a separate thiolated sample which was oxidized completely as described in the text. All melting points were determined at pH 7.

("disulfide interchange"). This experiment was therefore tried by adding a trace of β-mercaptoethylamine to a 10 per cent solution of intramolecular disulfide gelatin. As anticipated, this results in the rapid formation of a heat-stable gel.

DISCUSSION

In principle, the thiolation procedure reported here is applicable to any macromolecule with reactive aliphatic amino groups. In fact, even the insolubility of a polymer does not seem to limit its usefulness, since preliminary experiments have shown that aminated cotton[10] can also be substituted with N-acetylhomocysteine residues quite smoothly in a manner analogous to gelatin.

The introduction of as highly reactive a group as an —SH group into proteins obviously opens up a large number of possibilities. Coupling of other molecules with the newly built-in —SH groups could, of course, be easily accomplished, for example, by reaction with alkyl halides to form stable thioether bonds. In this way antigens, carcinogens, dyes, etc., could be firmly anchored to the protein mol-

ecule. The great reactivity of thiolated proteins with heavy metals deserves special mention. This property not only should be very useful for preparative work but would be of great advantage for the X-ray analysis of proteins by the isomorphous replacement method.[11]

The oxidation of the new —SH groups to disulfides provides, of course, an unequivocal way of introducing stable covalent cross-links into proteins. For example, the work reported here with disulfide gelatins bears out very clearly the correctness of the interpretation given by Huggins *et al.*[12] to their observations on the gelation of proteins in urea. In general, the deliberate cross-linking of proteins through disulfide bonds will permit a direct study of the effect of such alterations in the size and shape of proteins on their physical and biological properties.

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† Established investigator of the American Heart Association.

[1] R. Benesch and R. E. Benesch, *J. Am. Chem. Soc.*, **78**, 1597, 1956.

[2] R. Schwyzer and C. Huerlimann, *Helv. Chim. Acta*, **37**, 155, 1954.

[3] L. Weil and M. Telka, *Arch. Biochem. Biophys.*, **71**, 473, 1957.

[4] L. H. Noda, S. A. Kuby, and H. A. Lardy, *J. Am. Chem. Soc.*, **75**, 913, 1953.

[5] R. E. Benesch and R. Benesch, *J. Am. Chem. Soc.*, **77**, 5877, 1955.

[6] We are indebted to Dr. Paul Gallop for these measurements.

[7] A small proportion of a fast-sedimenting material is observed in the case of the disulfide gelatin, which is undoubtedly due to the formation of some intermolecular disulfide bonds.

[8] M. Calvin in *Glutathione* (New York: Academic Press, Inc., 1954), p.3.

[9] A. Fava, A. Iliceto, and E. Camera, *J. Am. Chem. Soc.*, **79**, 833, 1957.

[10] W. A. Reeves and J. D. Guthrie, *Textile Research J.*, **23**, 522, 1953.

[11] D. W. Green, V. M. Ingram, and M. F. Perutz, *Proc. Roy. Soc. London, A*, **225**, 287, 1954.

[12] C. Huggins, D. F. Tapley, and E. V. Jensen, *Nature*, **167**, 592, 1951.

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*THE INTERPRETATION OF BACTERIAL TRANSFORMATION DATA**

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The assay for bacterial transformation in *Pneumococcus* has been discussed critically by Hotchkiss.[1] He pointed out that a measure of the quality of a deoxyribonucleic acid (DNA) preparation is to be found in the number of transformants appearing at high DNA concentrations, when the bacterial sites are saturated. Since then, transforming DNA has been subjected to various physical treatments, such as sonication, fractionation, and ionizing radiation, and it appears desirable to review the problems that thereby arise in the interpretation of transformation data. It will be shown here that the changes in transforming ability which accompany the treatment of DNA cannot be simply assessed from either the plateau (saturated) region or the linear (low-[DNA]) portion of the transformants versus [DNA] curve. There have been a number of attempts at calculating the target size of the active unit from data obtained on the linear portion, without proper cognizance of all the variables, and it would appear that those results should be reassessed.