I. 2. THE INTRODUCTION OF NEW SULFHYDRYL GROUPS AND DISULFIDE BONDS INTO PROTEINS *

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I. Introduction

In contrast to the preceding paper which dealt with ways and means of dissecting proteins along their disulfide seams, this contribution is concerned with a method for grafting new sulfhydryl groups and disulfide bonds onto proteins. It seems virtually impossible at this time to devise methods by which cysteine residues could be introduced into the intact polypeptide sequences of proteins at desired intervals. On the other hand, attempts to introduce —SH groups into protein side chains by procedures which would not otherwise harm the protein molecule, seem worthwhile for two main reasons: (1) —SH groups are the most reactive groups encountered in proteins, and (2) direct oxidation of these groups leads to the most stable covalent cross-link found in proteins, i.e. disulfide bonds. The *de novo* introduction of these two forms of sulfur would thus provide an "anabolic" approach to the problem of the function of these important groups in naturally occurring proteins.

II. General Principles of the Thiolation Procedure

The method to be described in this paper is based on the use of thiolactones as thiolating agents. These compounds, which can be regarded as

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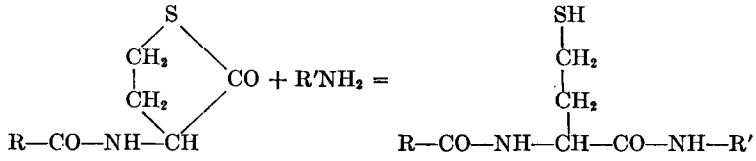
internal thiolesters, can react with amino groups with opening of the ring and the simultaneous formation of a peptide bond and a free —SH group. In the case of homocysteine thiolactone this type of reaction leads, under relatively mild conditions, to polymers of homocysteine, as was first observed by du Vigneaud *et al.* (*1*). We found subsequently (*2*) that *N*-acylhomocysteine thiolactones (in which the amino group was blocked to prevent this "autoaminolysis") react with the amino groups of other amino acids and peptides to form *N*-acylhomocysteinyl peptides:



The *N*-acetyl derivative seemed particularly promising for the thiolation of proteins because it is stable, easily prepared and purified and very soluble in water. *N*-acetylhomocysteinyl peptides could be prepared with this compound in *completely* aqueous solution at 0°. However, even at a total-NH_2 concentration of 0.1 *M* (which would be difficult to achieve with most proteins), a pH of 9–10 and reaction times of 15–20 hours were necessary to obtain good yields. These conditions were clearly not suitable for dealing with proteins. Recently, however, we discovered that in the presence of silver the reaction between *N*-acetylhomocysteine thiolactone (AHTL) and amino groups proceeds *rapidly at pH 7.5* at room temperature and still in completely aqueous solution. Schwyzer and Huerlimann (*3*) have reported a similar effect of silver on the aminolysis of certain thiolesters. It will be shown that this finding provides the basis for an extremely simple and effective way of thiolating proteins.

III. Experimental

When a solution of AHTL* is treated with one equivalent of $AgNO_3$, a precipitate is formed with the liberation of one proton per mole.† If the pH is kept constant at 7.5 by the simultaneous addition of NaOH, the precipitate dissolves slowly with the formation of the silver mercaptide of *N*-acetylhomocysteine. If the same experiment is carried out in the presence of one equivalent of glycine, the precipitate dissolves more rapidly with the disappearance of 30%–50% of the glycine amino groups. Since the main source of amino groups in proteins are the ϵ-amino groups of

* Prepared by the method described previously (*2*) or purchased from Schwarz Laboratories, Inc., Mount Vernon, New York.

† In the presence of excess silver, a maximum of 2 Ag per mole is bound and two protons are liberated.

lysine, the reactivity of this amino acid with AHTL in the presence of silver was investigated and it was found that the α- and the ϵ-NH_2 groups are substituted to about the same extent. The procedure was next tried on a high molecular polylysine.* One half of the free amino groups of this polymer could be easily substituted with *N*-acetylhomocysteine residues.

In applying these findings to proteins, gelatin was chosen because of its easy availability, and particularly, because it does not contain any significant amounts of cysteine or cystine. In general, the reaction with gelatin was carried out by treating an aqueous solution of gelatin† (3%–5%) and AHTL (cf. Table 1) with alternate increments of $AgNO_3$ and

TABLE I

THIOLATION OF GELATIN

Moles reagent per —NH_2 group	Groups per 10^5 gm.			
	Initial —NH_2[a]	Final —NH_2[a]	—NH_2 blocked	—SH formed[b]
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

[a] Determined by formol titration.

[b] Determined both according to P. D. Boyer, *J. Am. Chem. Soc.* **76**, 4331 (1954), and R. Benesch and R. E. Benesch, *Biochim. et Biophys. Acta* **23**, 643 (1957).

NaOH to keep the pH at 7.5, until a total of 1 mole of silver per mole of AHTL has been added. After about 1 hour a clear yellow solution is obtained. This 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.‡

* Kindly furnished by Dr. Elkan Blout, Polaroid Corporation, Cambridge, Massachusetts.

† The gelatin samples were kindly supplied by M. H. Rosenthal, Atlantic Gelatin, Woburn, Massachusetts. The two samples used for these experiments were a Type A gelatin (I.P. 6.95, Viscosity 18.0 m.p.) and a Type B Gelatin (I.P. 5.35, Viscosity 46.5 m.p.).

‡ Note added to proof: This procedure can be considerably shortened by precipitating the thiolated protein after the resin treatment with acetone, washing it with this solvent to remove all the thiourea, and drying it in a current of nitrogen.

Table I illustrates the close correspondence between the disappearance of amino groups and the appearance of sulfhydryl groups in the thiolated gelatins. This is an indication of the high specificity of the thiolating reagent for amino groups. The fact that in each case the —SH and —NH_2 groups add up to the amino group content of the parent gelatin shows that no significant degradation of the protein has taken place. It is also clear that the number of —SH groups introduced can be varied over a wide range simply by changing the ratio of reagent to protein.

IV. 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 II). Modification of the lysine ϵ-amino groups of proteins is known to reduce their susceptibility to tryptic hydrolysis (*4*). Table III shows this to be the case with thiolated gelatins.

TABLE II

ISOELECTRIC POINTS OF THIOLATED GELATIN

	I.P.[a]
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

[a] Isoelectric points were determined by the method of J. W. Janus, A. W. Kenchington, and A. G. Ward, *Research* **4**, 247 (1951).

TABLE III

TRYPTIC HYDROLYSIS OF THIOLATED GELATIN[a]

	Groups per 10^5 gm.	
Gelatin	Initial —NH_2	Maximum No. peptide bonds hydrolyzed by trypsin
Control	35.8	51
Thiolated	8.3	38

[a] 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^-) (*5*) and can therefore be used to determine the degree of ionization of —SH groups

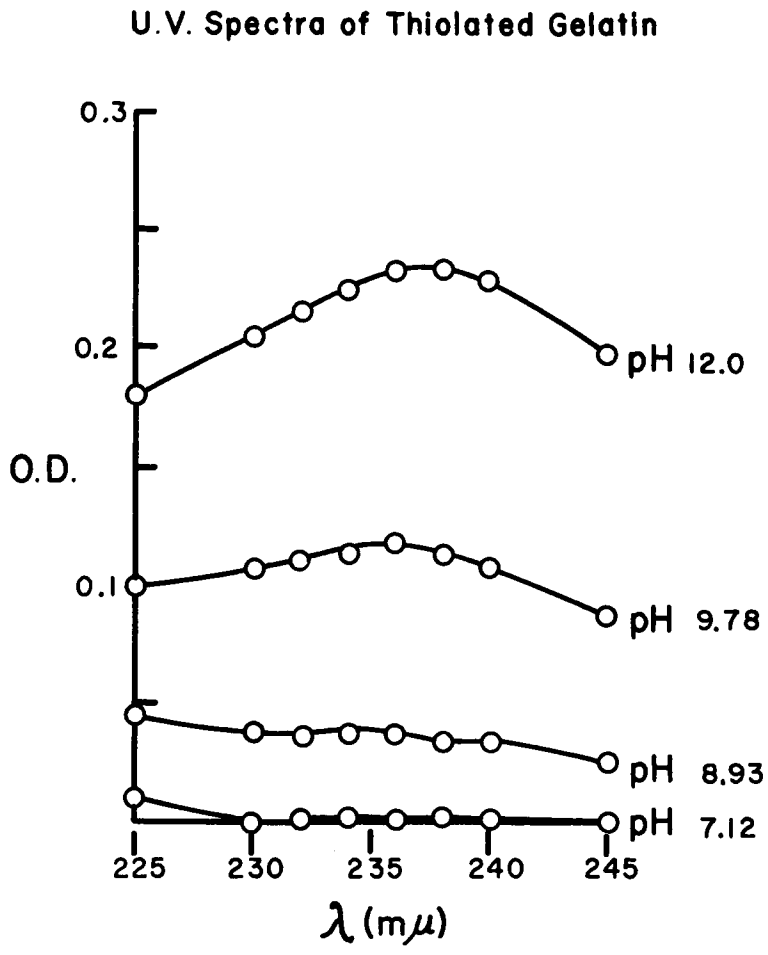


FIG. 1. These ultraviolet spectra of thiolated gelatin were obtained by methods previously described (*6*). Each spectrum represents the difference between the optical density at the given pH and the optical density of the same solution at pH 4.6.

(*6*). The effect of pH on the spectra of a gelatin containing 26 —SH groups per 10^5 gm. is shown in Fig. 1. When the —SH groups 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).

V. 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 Fig. 2.

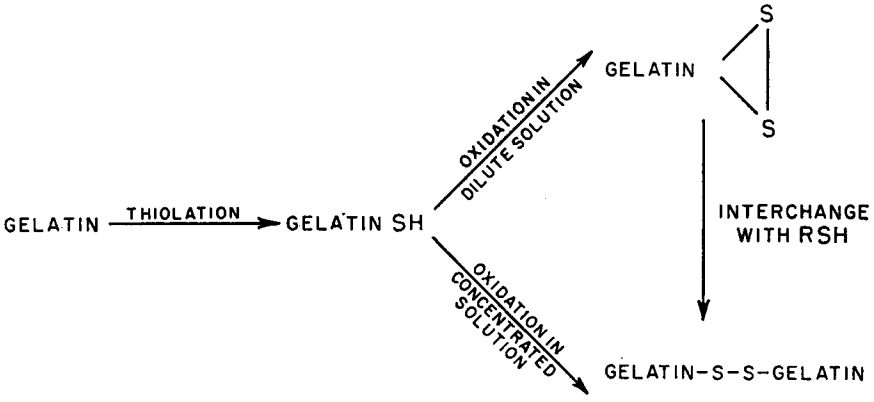


FIG. 2.

1. Oxidation in Dilute Solution (Intramolecular Disulfide Gelatin)

A 0.2% 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,* 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.†

* We are indebted to Dr. Paul Gallop for these measurements.

† 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.

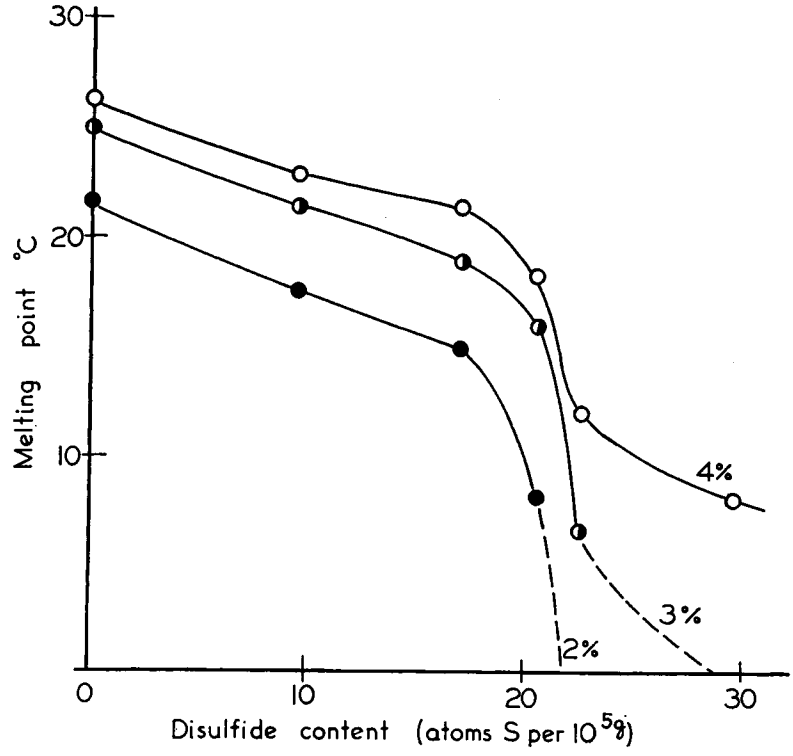


FIG. 3. Each point represents a separate thiolated sample which was oxidized completely as described in the text. All melting points were determined at pH 7.

These intramolecular disulfide gelatins show a dramatically reduced tendency to form gels. This is illustrated in Fig. 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%.

2. 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% or more, the solution sets almost instantly to a clear, colorless, rigid gel. These gels do not melt even at 100° 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 (*7, 8*) that intramolecular disulfide bonds can be changed to intermolecular disulfide bonds in the presence of catalytic amounts of thiols in alkaline solution ("disulfide interchange"). This experiment was therefore tried by adding a trace of β-mercaptoethylamine to a 10% solution of intramolecular disulfide gelatin. As anticipated, this results in the rapid formation of a heat-stable gel.

VI. Discussion

It seems worthwhile to list a number of the features of the method described here which make it particularly suitable for thiolating proteins:

(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.

(6) The number of sulfhydryl groups and disulfide cross-links introduced can be varied at will over a wide range.

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 (*9*) can also be substituted with *N*-acetylhomocysteine residues quite smoothly in a manner analogous to gelatin.

It is remarkable how little the thiolation *per se* affects the physical properties of the parent protein. This need not necessarily have been the case, since aggregation of proteins via —SH groups is known to occur (*10*).

It must be remembered that the —SH groups introduced by the present method are separated by eight more atoms from the polypeptide backbone than they would be in a cysteine side chain. It is therefore not surprising that these protein-linked —SH groups have the same acid strength as those of *N*-acetylhomocysteine itself.

Perhaps the most remarkable result of these investigations is the dramatic inhibition of gelation which occurs when a critical number of intra-

molecular cyclic disulfides is built into the gelatin molecule. This effect may either be due to drastic changes in the architecture of the protein towards a more globular shape or to direct obstruction of the sites which are concerned with the normal hydrogen bonding mechanism by which this protein forms gels.

The results with gelatin have certainly fulfilled our expectation that far reaching changes could be brought about in proteins by the introduction of sulfhydryl groups and disulfide bonds. Even though the new —SH groups per se did not appreciably affect the properties of gelatin, it remains to be seen how the biological activities of —SH enzymes such as aldolase, hormones such as insulin or heme proteins such as hemoglobin would be affected by built-in —SH groups. The oxidation of new —SH groups to intermolecular disulfides provides, of course, an unequivocal way of introducing stable, covalent cross-links into proteins. This should permit a direct study of the effect of such alterations in the size and shape of proteins on their physical and biological properties.

Acknowledgment

It is a pleasure to express our thanks to Dr. W. R. Middlebrook, who answered many justified and even unjustified questions with much patience and wisdom during the progress of this work.

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Discussion

HARRIS: I want to ask two questions. The first is, does the reaction always go with the ϵ-amino group?

BENESCH: Judging from experiments with lysine, the reaction proceeds to about the same extent with the α- and the ϵ-amino group.

HARRIS: Have you tried the reaction with an insoluble but swollen protein?

BENESCH: No, we have not.

HARRIS: I think it will go. I do not know about accessibility but in similar materials, the epsilon amino groups act as though they are in solution.

BENESCH: We have tried it with cotton and it works very well with that. If you take aminated cotton you can thiolate it very well with this reagent. It does make a definite difference whether or not you pre-swell cotton.

HARRIS: The extent of the reaction will depend on that.

BENESCH: Exactly.

SWAN: Would Dr. Benesch care to comment on possible mechanisms for this amine-silver ion-thiolester reaction?

BENESCH: The only explanation which I can offer is the concept from which we started, namely that silver on the one hand activates the amino group by complexing it and also has a high affinity for the —SH group formed as a product of the reaction.

HARRIS: Have you tried the effect of proteolytic enzymes on your materials which have been oxidized from solutions of various concentrations? You know the old dilution principle: if you take wool and reduce it to a soluble form and then reoxidize it in a concentrated solution, you will have a system which is still resistant to attack by the proteolytic enzymes.

BENESCH: We have not done that.

BROWN: I would like to ask a question about thioglycolide. As you probably know, it has been used by others to put SH groups into proteins. Since you have a fairly long alkyl chain, and gelatin as the substrate, it might be interesting to see what thioglycolide would do under the same conditions. Have you done anything on that?

BENESCH: No we have not, since the compounds as described by Schöberl were so ill-defined that we gave up the idea of going any further with thioglycolide. Do you have some thioglycolide?

BROWN: Yes.

BENESCH: Other than residues from old bottles of thioglycolic acid?

BROWN: You answered the question I was going to comment on. An easy way to get thioglycolide is to set 99% thioglycolic acid on the shelf and wait two months.

BENESCH: Only two months?

BROWN: Maybe one month or so. A lot of people think it is impure but we have taken the solid material out and indeed that is thioglycolide.

BENESCH: Yes, but in what state of polymerization?

BROWN: I agree with you one hundred per cent that these are ill-defined compounds.