Estimation of Cystine plus Cysteine in Proteins by the Disulfide Interchange Reaction*

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The interchange reaction between disulfides in concentrated acid solutions was demonstrated by Sanger (1) in 1953 with model compounds. Ryle and Sanger (2) later studied the formation of mono-dinitrophenyl cystine peptides in the reaction between insulin and di-DNP-cystine in order to estimate the rate and extent of the interchange reaction. The disulfide interchange reaction was studied from the standpoint of mechanism by Benesch and Benesch (3). They concluded that the mechanism could be represented by the following scheme:

Initiation: \[ RSSR + H^+ \rightarrow RS^+ + RSH \]  (1)

Interchange: \[ RS^+ + R'SSR' \rightarrow RSSR' + R'S^+ \]  (2)

Inhibition: \[ R'S^+ + RSH \rightarrow RSSR' + H^+ \]  (3)

Ryle and Sanger (2) observed that the reaction was very slow at hydrochloric acid concentrations below 9 N, but showed only a slight dependence on acid concentration above this value. Both Ryle and Sanger (2) and Benesch and Benesch (3) were able to show that the rate of the interchange reaction was greatly decreased by thios.

The present paper describes the use of the interchange reaction for the estimation of the cystine plus cysteine content of proteins. Briefly, the procedure involves the following steps: (a) incubation of protein with an excess of di-DNP-cystine in strong hydrochloric acid at 39°, (b) withdrawal of aliquots at intervals, (c) extraction of di-DNP-cystine from these samples with ether, and (d) spectrophotometric determination of mixed disulfide remaining in the aqueous acid solution after the ether extraction.

The method has given reproducible results with many proteins. For proteins with known disulfide and sulfhydryl content, the agreement of the values obtained with the published values is excellent. It may be noted that in the studies of Benesch and Benesch (3) and Ryle and Sanger (2), either cystine or protein (in terms of its disulfide content) was in excess of the di-DNP-cystine.

In the present work, this situation has been reversed, a considerable excess of di-DNP-cystine compared to the cystine content of the reaction mixture being maintained.

**Experimental Procedure**

**Materials and Methods**

**Materials**—N, N'-Bis-2,4-dinitrophenyl-L-cystine (di-DNP-cystine) was prepared by the method of Porter and Sanger (4) and its purity checked chromatographically. N-2,4-Dinitrophenyl-L-cystine was prepared as described by Ryle and Sanger (2). Its molar extinction coefficient in 6 N HCl is 15,000 at 357 mp, and its purity was determined chromatographically.

Cysteine was an analytical reagent obtained from Winthrop-Stearns, Inc. Cysteine-HCl, analytical reagent grade, was obtained from Nutritional Biochemicals Corporation and contained 89% sulfhydryl by the N-ethylmaleimide and p-chloromercuribenzoate methods. Glutathione (Schwartz BioResearch, Inc.) contained 98% sulfhydryl as determined by the p-chloromercuribenzoate and N-ethylmaleimide methods. Analytical reagent grade HCl was used.

**Procedure**—A weighed amount of the protein preparation was dissolved in a volume of 9.6 N HCl such that the concentration of protein was in the range 0.025 to 0.20 μmoles per ml. The solution was then divided into two equal parts. One part represented the blank. To the other part, 1.2 mg of di-DNP-cystine per ml² were added, and the vials were closed tightly and placed in a water bath at 39° ± 0.03°, unless otherwise specified. Before immersion, the vials were wrapped with aluminum foil. Small glass vials with polyethylene covers which provided an airtight seal were used throughout. After immersion for several hours in the bath, the aluminum foil adhered tightly to the glass, forming an excellent protection against light. Samples were withdrawn from the reaction mixture at the time intervals desired.

Aliquots of 1 ml were withdrawn and pipetted directly into vials containing 1.5 ml of water and 3.0 ml of ether. The di-DNP-cystine was then extracted once with 6 ml, and three times with 3 ml of ether. The ether present in the aqueous phase was evaporated by gentle suction, and the aqueous phase was transferred quantitatively to a 25-ml volumetric flask and diluted to the mark with 6 N HCl.

The use of 6 N HCl was necessary in cases in which it was desired to study the rate of the reaction within 6 to 18 hours of the start of the experiment. In the case of some proteins, turbidity was avoided by the use of a saturated solution of di-DNP-cystine to compensate exactly for the amount of di-DNP-cystine used in the interchange reaction.

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1 The following abbreviation is used: DNP, dinitrophenyl.
bidity was observed when the aqueous phase was diluted with water or HCl at concentrations lower than 6 N.

The absorbancy (A) at 357 mμ in a 1-em cell of the diluted aqueous phase, and of the blank which had been treated in exactly the same manner, was then determined. Either a Beckman DU or a Cary model 14 spectrophotometer was used. The calculation is then simply: μmoles of mono-DNP-cystine per ml of reaction mixture = 25 A/15 = 1.67 A.

The interchange reaction was generally followed until the mono-DNP-cystine concentration in the aqueous phase did not change by more than 1% over a period of 4 days. When the reaction approached equilibrium, the determinations of mono-DNP-cystine were carried out in triplicate, and the values obtained were averaged.

**Determination of Protein Content**—In most cases, protein concentration was determined by using the known extinction coefficient of the protein in question in the region of 280 mμ. For maximal precision, the extinction coefficient of the protein in 6 N HCl was compared with that of a neutral solution at the same concentration of protein. The correction involved was of the order of 2 to 5%, the absorbancy being less in all cases in the 9.6 N HCl than in the neutral solution. Once the extinction coefficient in 9.6 N HCl was available, a direct determination of the protein concentration in the reaction mixture blank could be performed. It was found for all the proteins studied that when such a determination was performed within 30 minutes of dissolving the protein in 9.6 N HCl, no measurable loss of tyrosine or tryptophan had taken place at room temperature. In a few cases, when values of Eᵇ, were not available, the protein content was obtained by performing dry weight determinations and correcting for the moisture content of the protein.

In general, it was found that deionized proteins gave lower blank values, and the use of such preparations is advised if high accuracy is desired.

Allquots were removed from reaction mixtures containing different proteins and di-DNP-cystine at different times during the interchange reaction and after equilibrium was reached. These samples were extracted with ether as described above. Two-dimensional chromatography was performed with the "toluene" system (5) in the first dimension and sodium citrate in the second dimension (6). Pyridine-acetic acid-water, at pH 0.4, was used for the electrophoresis (7) and n-butanol-acetic acid-water (200:30:75) for the chromatography in the two-dimensional electrophoresis-chromatography. Examination of the chromatograms after both these separations revealed that the ether phase contained only di-DNP-cystine. No other DNP derivatives or ninhydrin-positive material could be detected. The aqueous phase contained no trace of di-DNP-cystine.

No breakdown of di-DNP-cystine was observed after 4 weeks at 39° or after 48 hours at 60° in 9.6 N HCl. After more than a few days at 60°, some breakdown of di-DNP-cystine was observed. Since the concentration of the mono-DNP-cystine peptides present at equilibrium (as determined spectrophotometrically) remained constant over a considerable period of time, it is apparent that the mono-DNP-cystine is also stable under the conditions used. It should be emphasized that the reactions were studied with all light excluded, since it is well known that these DNP derivatives are light-sensitive.

**General Observations and Optimal Conditions**

In agreement with the findings of Ryle and Sanger (2), it was observed that the rate of interchange in 12 N HCl was essentially the same as that in 9.6 N HCl.

"Aging" of Di-DNP-cystine Solution—Renoux and Renoux (3) observed that the interchange reaction proceeded at a faster rate when either or both of the participants had been allowed to stand for a long period of time in 12 N HCl. They considered this effect to be due to the accumulation of RS+.

A saturated solution of di-DNP-cystine in 12 N HCl was allowed to stand in the dark for 2 months. The rates of interchange obtained with this reagent and a freshly prepared one were then compared in the case of several proteins. Although the initial rate of interchange was somewhat increased, this effect disappeared after several hours, and the rate became identical to that observed with the freshly prepared reagent.

**Effect of Temperature on Rate of Interchange**—It was found that when the interchange reaction was carried out at 60°, the rate of the reaction was appreciably increased. There are, however, two disadvantages to using this high temperature. First, the equilibrium concentration of mixed disulfide for a given protein concentration was approximately 10% less than that obtained at 39°, thus lowering the precision of the measurements. Secondly, some destruction of di-DNP-cystine took place at 60°, whereas no detectable destruction of the reagent could be observed at 39°. In view of these findings, it was considered that the disadvantage of the longer time taken to attain equilibrium at the lower temperature was offset by the advantage of greater precision.

**Reaction with Thiol and Effect of Added Cysteine**—The interchange reaction occurred with both cysteine and glutathione, although at a very much slower rate than with either cysteine or ribonuclease (Fig. 1).

When added to a disulfide, such as cystine, cysteine was found to slow down the rate of the interchange reaction considerably (Fig. 1). The same effect was observed on addition of cysteine to a protein containing only disulfide bonds, e.g. insulin. The extent of decrease in rate of the interchange depended on the relative amount of protein and cysteine. It should be noted that when sufficient time was allowed for the mixture to attain equilibrium, i.e. several weeks, the final value of mixed

![Fig. 1. Reaction of thiol and disulfide-containing compounds with di-DNP-cystine in 9.6 N HCl at 39°. □, cystine (0.6 μmole per ml); ○, cysteine (1.6 μmole per ml); ●, glutathione (1.6 μmole per ml); △, cystine (0.6 μmole per ml) plus cysteine (0.4 μmole per ml).](http://www.jbc.org/)
Rate and Equilibrium of Interchange Reaction—The rate at which equilibrium was attained depended markedly on the protein under investigation. Of the proteins studied, ribonuclease was found to reach equilibrium most rapidly, within 48 hours, whereas trypsin attained equilibrium only at the end of approximately 21 days. Some data for several representative proteins are presented in Figs. 2 and 3.

In the half-cystine concentration range of 0.1 to 3.4 μmoles per ml of reaction mixture, it was observed that as the concentration of half-cystine (i.e., protein) was increased, the amount of the mixed derivative per mole of half-cystine, obtained at equilibrium, was diminished (Fig. 3). This is probably due to the fact that as the absolute concentration of the mixed derivative becomes comparable with that of the di-DNP-cystine, the reverse reaction leading to the formation of symmetrical disulfide becomes important.

\[ R-S-S-R' + R'S^+ \rightarrow R'-S-S-R' + RS^+ \]

This observation was utilized for the determination of the half-cystine content of proteins. The amount of mono-DNP-cystine present at equilibrium was determined at several protein concentrations for each protein, and the ratio of μmoles of mono-DNP-cystine per μmole of protein was calculated for each concentration of protein. The values thus obtained were plotted against the protein concentration, and the line through these points, calculated by the method of least squares, was extrapolated to zero protein concentration. The intercept represents the limiting number of mono-DNP-cystine residues and, hence, the total cysteine and half-cysteine content of the protein. Several such representative plots are shown in Fig. 4.

Table I gives for a number of proteins the half-cystine values obtained by the extrapolation method described above. It is evident that for these proteins, the values determined by this method are in excellent agreement with the published values for the content of half-cysteine plus cysteine. For the sake of brevity, only a few of the more recent values from the literature are cited.

The equilibrium values for the mono-DNP-cystine formed may be plotted as a function of the total cysteine plus half-cysteine content of the reaction mixture. Such a plot is given in Fig. 5 for bovine serum albumin, trypsin, and ribonuclease. It is evident that the points fit on a single smooth curve and, indeed, data obtained with other proteins also fit the same curve very well.

Inasmuch as the data for all proteins studied fit on the same curve, it becomes possible to determine the half-cysteine content of a protein on the basis of a single determination, provided that a curve such as the one in Fig. 5 has been constructed, or simply by using the curve in Fig. 5. The most precise values are obtained when the half-cysteine content of the reaction mixture is in the range of 0.5 to 1.5 μmoles of half-cysteine per ml. It should be emphasized, however, that the values obtained from a single determination are inevitably less reliable than those obtained by the extrapolation method.

In Table II some results are given for several proteins. In these instances, duplicate determinations of the equilibrium values for the mono-DNP-cystine formed at a single protein concentration were performed. The results were then calculated by interpolation on the smooth curve of Fig. 5. The data in Table II indicate that satisfactory results can be obtained by this method.

Specificity of Reaction—No reaction with any amino acid other than cystine or cysteine could be observed under the conditions described above. Methionine did not react at all, regardless of the concentration used. Proteins known to be free from cystine and cysteine, e.g. whale myoglobin and protamine sulfate (salmine), did not give any interchange (Table II). Perhaps the strongest evidence supporting the view that the interchange reaction is completely specific for cystine (and cysteine), under the conditions used in these experiments, is the fact that the results obtained with proteins of known sulfur content are in complete accord with those reported in the literature.
TABLE I

Half-cystine plus cysteine content of proteins

These values were determined by extrapolation to zero protein concentration by plots similar to those given in Fig. 4. The values were calculated by the method of least squares.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>$e^{*1}$ cm.</th>
<th>Assumed mol. wt.</th>
<th>Half-cystine + cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin (crystallized)</td>
<td>Armour Lot No. 128-176</td>
<td>6.60 (8)</td>
<td>69,000</td>
<td>34.9 ± 0.5</td>
</tr>
<tr>
<td>Trypsin (crystallized)</td>
<td>Worthington Biochemical Corp. Lot No. TRSF-688</td>
<td>14.1 (10)</td>
<td>24,000</td>
<td>12.0 ± 0.2</td>
</tr>
<tr>
<td>Ribonuclease (crystallized)</td>
<td>Worthington Biochemical Corp. Lot No. 558</td>
<td>6.9 (12)</td>
<td>12,683</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>Insulin (bovine)</td>
<td>Squibb, Zn-free</td>
<td>10.0 (14)</td>
<td>5,700</td>
<td>5.9 ± 0.1</td>
</tr>
<tr>
<td>Ovalbumin (crystallized)</td>
<td>Nutritional Biochemical Corp., twice crystallized, salt-free</td>
<td>7.35 (16)</td>
<td>45,000</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>Lysozyme (crystallized)</td>
<td>Armour Lot No. 003L1</td>
<td>27.3 (18)</td>
<td>14,000</td>
<td>10.4 ± 0.4</td>
</tr>
</tbody>
</table>

*Note: Assumed mol. wt. values were obtained by interpolation on the standard curve of Fig. 5.

DISCUSSION

When this investigation was initiated, it was hoped that it would be possible to distinguish between disulfide and sulfhydryl content, or at least between proteins containing only disulfide bonds and those containing both disulfide bonds and sulfhydryl groups, on the basis of the rates of the interchange reaction. It is evident from inspection of the figures that this did not prove to be possible. Thus, for example, trypsin which contains six disulfide bonds and no sulfhydryl groups reacts with di-DNP-cystine in the interchange reaction far more slowly than ovalbumin which has only one disulfide bond and four or five sulfhydryl groups.

Under the conditions used in this study, the proteins may be considered to undergo essentially complete denaturation immediately on coming in contact with the 9.6 N HCl. Indeed, the proteins also undergo extensive hydrolytic cleavage within a short time. It is clear, therefore, that differences in the reactivity of the disulfides in different proteins cannot be ascribed to effects based on the presence of secondary structure. Rather, the explanation probably resides in the influence of neighboring polar groups in the peptides containing the disulfide. Such polar groups may either labilize the S-S bond or render it less active. Part of the effect exerted by the neighboring groups may, of course, be due to repulsion of the positively charged attacking ion (RS+) and also of protons when positively charged e-ammonium groups of lysine or guanidinium groups of arginine are in close proximity to the disulfide bond.

TABLE II

Half-cystine plus cysteine content of proteins

These values were determined by duplicate estimations of mono-DNP-cystine formed at a single protein concentration. The protein content of half-cysteine plus cysteine was then estimated by interpolation on the standard curve of Fig. 5.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>$e^{*1}$ cm.</th>
<th>Assumed mol. wt.</th>
<th>Half-cystine + cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin (porcine)</td>
<td>Crystallized 3 times</td>
<td>14.3 (22)</td>
<td>35,500</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td>α-Lactoglobulin</td>
<td>Crystallized 3 times</td>
<td>9.5 (24)</td>
<td>35,500</td>
<td>9.8 ± 0.2</td>
</tr>
<tr>
<td>Chymotrypsinogen (crystallized)</td>
<td>Armour R3372S</td>
<td>20.0 (26)</td>
<td>35,100</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td>Myoglobin, whale (recrystallized)</td>
<td>Gift of Dr. J. C. Kendrew</td>
<td>17,000</td>
<td>100,000</td>
<td>35.1 ± 0.8</td>
</tr>
<tr>
<td>Human γ-globulin (II, 1, 2)</td>
<td>Squibb</td>
<td>8,000</td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>Squibb</td>
<td></td>
<td></td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Protein content was determined by dry weight.
† This value is based on a molecular weight of 100,000 and a half-cysteine plus cysteine content of 2.6% calculated from the total sulfur minus the methionine sulfur of this preparation.
The importance of electrostatic effects has been shown by Moore and Porter (30), who studied the exchange reaction of aranesulfenyl chlorides (e.g., 2,4-dinitrophenylsulfenyl chloride) with organic disulfides. These investigators demonstrated that the rate of exchange at room temperature was dependent on the polarity of the solvent, exchange being considerably faster in acetic acid than in less polar solvents, such as ether, carbon tetrachloride, and xylene.

The differences in the rate of interchange found with the various proteins under conditions in which all secondary structure and much of the primary have been destroyed, illustrate the great importance of the neighboring amino acids in the peptide chain in determining the reactivity of the disulfide bond.

In view of the mechanism proposed by Benesch and Benesch (3) for the interchange reaction, the interchange obtained with thiols may appear to be unexpected at first glance. It should be noted, however, that no attempt was made in the present studies to exclude either oxygen or trace metals from the reaction mixture. Hence the reaction observed may be due to slow oxidation of thiol groups to disulfides, which can then participate in the interchange reaction. The above interpretation is also consistent with the finding that for proteins such as \( \beta \)-lactoglobulin, ovalbumin, etc., which contain both disulfide and thiol groups, the values obtained from the interchange reaction represent the total of the disulfide and sulfhydryl content.

It should be mentioned that if sulfenic, thiol ester, or thiazoline groups exist in proteins, and if these groups are capable of either reacting directly with di-DNP-cystine, or giving rise to sulfhydryl and, hence, disulfide under the conditions of the interchange reaction, such groups would be included in the mixed disulfide value as determined by this method.

The agreement of the values obtained in this study with those reported in the literature is excellent. In the case of \( \gamma \)-globulin, the value of 33.1 ± 0.8 obtained for the cystine plus cysteine content of this protein agrees well with the value of 34.6 calculated from the total sulfur content minus the methionine sulfur present in this protein. The data in Fig. 4 show that for proteins with three to six disulfide bonds (e.g., insulin, ovalbumin), two or three points suffice to obtain a reliable extrapolated value. Therefore, it is possible to obtain such a value with as little as 0.6 to 1 mmole of protein.

Although earlier work had indicated that egg white lysozyme contains 10 half-cystine residues (20, 21), Jolles et al. (19) have indicated recently that this protein may contain only eight such residues. It is noteworthy that our results indicate 10 half-cystine residues. It is noteworthy that our results indicate 10 half-cystine residues of this protein. The results obtained with other proteins have been in good agreement with those in the literature for the half-cystine plus cysteine content of 12 different proteins. The equilibrium values of the reaction were utilized in the development of a method for the precise estimation of the half-cystine plus cysteine content of proteins. The results obtained are in good agreement with those in the literature for the half-cystine plus cysteine content of 12 different proteins.

SUMMARY

The disulfide interchange reaction of di-dinitrophenyl cystine in concentrated acid solution with cystine, simple thiols, and a number of proteins has been investigated. The effects of acid concentration, temperature, and protein concentration on the kinetics and equilibrium of the exchange reaction have been studied. The reaction appears to be specific for the disulfide and thiol groups of proteins. No reaction with methionine or with proteins lacking sulfur-containing amino acids could be demonstrated. The various proteins studied varied widely with respect to the rate of exchange with di-dinitrophenyl cystine. The equilibrium values of the reaction were utilized in the development of a method for the precise estimation of the half-cystine plus cysteine content of proteins. The results obtained are in good agreement with those in the literature for the half-cystine plus cysteine content of 12 different proteins.

REFERENCES


\[ 3 \text{ A. N. Glazer and E. L. Smith, in preparation.} \]
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