SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

I. Methods of Estimation

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INTRODUCTION

There have been no methods for estimating quantitatively the sulfhydryl (SH) and disulfide (S-S) groups of proteins. And yet enough is known of these groups to indicate their importance from several points of view. SH and S-S groups appear in proteins during coagulation, and their appearance is the only change known to occur in the constitution of the protein on coagulation (Heffter, 1907; Arnold, 1911). They may be of interest for an understanding of the chemical changes which take place in muscle; SH groups can be detected in the proteins of minced muscle even before the tissue has been treated with a coagulating agent (Arnold, 1911). SH groups are also significant for the study of enzymes for in the case of crystalline urease it has been observed that activity depends upon the presence of these groups (Sumner, 1933).

In this paper methods for estimating protein SH and S-S groups are described. It is shown for the first time that the number of SH and S-S groups detectable in a coagulated but unhydrolyzed protein is equivalent to the quantity of cysteine and cystine found in the hydrolyzed protein. In native proteins, on the other hand, only a small fraction of these groups can be detected. To develop methods for estimating protein SH and S-S groups we have used denatured proteins because the validity of the method can best be tested on such preparations. The methods are also applicable to native proteins, however, and the results obtained for various native proteins will soon be published.

We have developed two independent methods for estimating the reactive SH groups of unhydrolyzed protein. In the *direct* method the SH groups are oxidized by cystine, which is thereby reduced to cysteine, and the number of protein SH groups oxidized is estimated by measuring the amount of cysteine formed. Cystine oxidizes all the SH groups and no other groups in the protein. In the *indirect* method SH groups are eliminated by treatment with an oxidizing agent or with iodoacetate, and the reagent added is removed. The protein is then hydrolyzed, its total cysteine content is estimated and compared with the cysteine content of untreated protein. The diminution in cysteine content is equal to the number of SH groups which reacted with the reagent. Instead of measuring the cysteine contents of hydrolysates one can estimate the SH groups of the unhydrolyzed treated and untreated protein by the reaction with cystine.

The number of S-S groups in unhydrolyzed protein is estimated by the increase in number of SH groups caused by reducing the S-S groups to SH with thioglycollic acid.

The cystine and cysteine contents of protein hydrolysates are estimated colorimetrically by their reactions with phosphotungstate. Cysteine, but not cystine, gives a blue color with phosphotungstate in the absence of sulfite. Cystine gives a blue color in the presence of sulfite which half reduces it to cysteine. To measure the cysteine content of a protein, therefore, phosphotungstate is added to the hydrolysate in the absence of sulfite. To measure the total cysteine plus cystine content, all the cysteine is oxidized to cystine, and the color reaction with the hydrolysate is carried out in the presence of sulfite. For the estimation of total cysteine plus cystine our method is derived from that of Folin and Marenzi (1929). There has hitherto been no method for estimating the cysteine content of proteins.

HISTORICAL

Protein SH groups have usually been detected by means of the color reaction they give with nitroprusside in presence of dilute ammonia. It was by this color test that they were first detected in coagulated egg albumin by Heffter and by Arnold. Using the same test, Arnold made a systematic investigation of the substances in tissues which contain SH groups and observed that these groups are present in two distinct forms—in the protein of tissues and in protein-free

extracts of tissues. His experiments led him to believe that the substance in tissue extracts which contains SH groups is cysteine. 20 years later Hopkins isolated this substance and showed it to be not cysteine but a peptide containing cysteine, which he named glutathione (Hopkins, 1921, 1929). There have been many investigations of glutathione but very few of proteins as SH compounds.

Heffter supposed that many denatured proteins, the denatured serum proteins for example, which do not give a color reaction with nitroprusside and hence do not contain SH groups, have S-S groups which can be reduced to the SH form. By treating the denatured serum proteins with the reducing agent, sodium sulfite, he caused them to give a reaction with nitroprusside. Protein S-S groups have also been reduced by means of tin and hydrochloric acid (Arnold, 1911) by soluble SH compounds (Hopkins, 1925), and by cyanide (Walker, 1925).

Protein SH groups can be oxidized to the S–S form. For this purpose Heffter used sulfur which removes the hydrogen from protein SH groups and converts it into hydrogen sulfide. Heffter attempted to estimate protein SH groups by measuring the amount of hydrogen sulfide formed. A soluble disulfide, oxidized glutathione, was later used by Hopkins instead of sulfur. His experiments demonstrated clearly the existence of equilibria between glutathione in solution and sulfur groups in the protein. No more than this was claimed for them (Hopkins, 1925).

Direct Estimation of Protein SH Groups

This method is based on the principle first used by de Rey Pailhade (1888) and Heffter, and later by Hopkins. The hydrogen of the protein sulfur groups is transferred to another sulfur compound and then estimated. For this purpose we have allowed the protein to react with cystine and have then estimated the quantity of cysteine formed. If this amount of cysteine is to be considered a measure of the number of SH groups present in the protein, it must be shown that *all* the protein SH groups react with cystine and *only* these. That the protein SH is completely oxidized by cystine is shown by the fact that the treated protein does not give any color with nitroprusside, a sensitive reagent for SH. That only SH is oxidized is indicated by the fact that only those proteins reduce cystine which give a color with nitroprusside, a fairly specific reagent for SH. Both the completeness and specificity of the oxidation are shown by the fact that the number of cystine molecules reduced by a completely reduced protein is equivalent to the number of cystine molecules found in the hydrolysate of the same protein when completely oxidized.

Estimation of the cysteine formed by action of protein SH groups was first attempted by titration with iodine. This method proved inaccurate due to the low concentration of cysteine in the solutions. It was then found that cysteine in low concentrations can be estimated accurately by means of the color it develops with phosphomolybdate or phosphotungstate. Phosphotungstate has been used by Folin and Looney for the estimation of cystine.[1] In their method the color is developed in an alkaline medium and in presence of an excess of sodium sulfite which reduces the cystine and also prevents fading of the color. For our purpose sulfite could not be used to prevent fading because of its effect upon cystine. A solution of the difficulty was provided by the observation that cysteine develops a blue color with phosphomolybdate or phosphotungstate even in neutral solutions and that in these solutions fading does not occur in the absence of sulfite. Under these conditions a satisfactory proportionality over a wide range is obtained between concentration of cysteine and intensity of color. In a volume of 25 cc. the quantity of cysteine can vary from 0.4 mg. to 1.0 mg. with a proportionate change in color intensity. A similar method for the estimation of cysteine was published by Lugg (1932) after most of the experiments described in this paper had been completed.

The properties of cystine impose some limitations on the method for estimating protein SH groups by oxidation with cystine. One difficulty is that cystine is insoluble at the neutral point and becomes sufficiently soluble only when the pH is more than 9.0. Furthermore, for investigating the activity of SH groups under various conditions, a stronger oxidant than cystine is desirable. When ferricyanide, a more powerful oxidant is used, other reducing groups of proteins in addition to the SH are sometimes oxidized.[2] Indeed, one reason that the reduction of cystine by proteins is a specific test for protein SH groups is that cystine is a very mild oxidant. Strong oxidizing agents can be used in the estimation of protein SH groups if the change occurring in the protein itself is examined rather than the change in the

[1] Folin and Looney did not estimate the amount either of cystine by itself or of cysteine in the presence of cystine.

[2] An investigation of some other reducing groups of proteins will be published in another paper.

oxidizing agent, as is done when cystine is used. We have devised another method based on this principle.

Indirect Methods for Estimating Protein SH Groups

In these methods protein SH groups are oxidized or they are blocked with iodoacetate, the excess of oxidizing agent or iodoacetate is removed, and the number of SH groups remaining in the protein is compared with the number originally present.

Hydrogen peroxide and potassium ferricyanide[3] can be used as oxidizing agents. Peroxide is easier to use and is especially useful if the cystine content of oxidized protein is to be estimated. Iodoacetate reacts with SH groups according to the equation:

$$RSH + CH_2I\ COOH \rightarrow RS - CH_2\ COOH + HI$$

To estimate the number of protein SH groups before and after treatment with hydrogen peroxide or ferricyanide the protein can be exposed to cystine as described above.[4] An entirely different method, involving hydrolysis of the protein is more convenient. The protein is hydrolyzed, and the SH groups in the hydrolysate are estimated by the color developed with phosphotungstate. The color reaction is carried out as described above for the estimation of cysteine except that a large quantity of urea is added to prevent the formation of a precipitate. If a large number of SH groups were uncovered by hydrolysis this method would not be accurate. Actually no such uncovering on hydrolysis takes place. When the SH groups of a denatured protein are oxidized or blocked, no groups at all are found even after hydrolysis. It might be supposed that protein SH groups would be oxidized during acid hydrolysis, and some support for this view is provided by the fact that if cysteine be added to the protein during hydrolysis, not all of it will be recovered. And yet it can be shown that protein SH groups are not oxidized during the acid hydrolysis used:

1. Estimation of protein SH groups by measurements of the SH contents of the hydrolysates of reduced and untreated proteins agrees

[3] The use of potassium ferricyanide will be described in a paper on the SH groups of hemoglobin.

[4] This procedure has not been tried after blocking SH groups with iodoacetate.

with the results obtained by the cystine method which involves no hydrolysis.

2. In the case of proteins of the crystalline lens which contain cysteine and no cystine the number of SH groups estimated after hydrolysis is equivalent to the quantity of cystine found in the hydrolysate of an oxidized preparation.

Oxidation of SH groups while the protein is being dried preparatory to being weighed and hydrolyzed must be avoided. If neutral protein is dried at 105°C., some oxidation of its SH groups occurs. This can be prevented by keeping the protein acid while it is being dried and by shortening the time necessary for drying. For these reasons the protein is dehydrated with acid acetone before drying.

Estimation of S-S Groups

In the estimation of protein S-S groups these groups are reduced to SH groups; the increase in SH groups is equal to the number of S-S groups originally present. The properties required of a reducing agent are that it should completely reduce S-S groups and that any excess of it remaining after reduction should be completely removable since the reducing agent may react with the reagent used to estimate SH.

Sodium hydrosulfite and sodium sulfite do not completely reduce protein S-S groups. Indeed, when cystine is treated with sodium sulfite, as will be shown below, even in presence of a great excess of the reductant, exactly 50 per cent of the cystine is reduced. Protein S-S groups are reduced by thiol compounds, such as cysteine, reduced glutathione, and thioglycollic acid. Here an equilibrium is established.

$$\text{Protein S—S} + 2\,R\text{SH} \rightleftharpoons \text{Protein } \underset{\text{SH}}{\text{SH}} + R\text{S—S}R$$

Of these thiol compounds, thioglycollic acid is the least expensive and also the most effective reducer of protein S-S groups. After a certain concentration of thioglycollic acid has been reached, doubling the concentration does not result in a further increase in number of protein SH groups. This fact indicates that the reaction has gone to completion, but does not prove that the S-S groups have been com-

pletely reduced. In the case of reduction of cystine by sulfite, it was such evidence that led Folin and Looney to believe that cystine was completely reduced when it was in fact only one-half reduced, while the other half underwent some other change. To show that reduction by thioglycollic acid was actually complete, the maximum possible number of S-S groups in an oxidized protein was determined by hydrolyzing it and estimating the cystine content of the hydrolysate. This number corresponds exactly to the number of SH groups present after reduction by thioglycollic acid in the denatured but unhydrolyzed protein. Since this correspondence between number of cystine molecules and number of SH groups holds for denatured proteins, in these proteins reduction of S-S groups by thioglycollic acid is complete. That there is an excess of SH groups after reduction indicates that no thioglycollic acid remains adsorbed to protein, for any SH groups due to adsorbed thioglycollic acid would be included in the estimation of protein SH groups.

The Cystine and Cysteine Content of Proteins

In attempting to use the Folin-Marenzi method for estimating the cystine content of proteins, we found that it can give results as much as 100 per cent too high. In this method cystine in a protein hydrolysate is reduced with sulfite, and the cysteine formed is estimated by means of the blue color which it produces with phosphotungstate. Folin and Looney believed they had shown, as has been mentioned, that the reduction of cystine by sulfite is complete and that consequently any cysteine present in the protein hydrolysate could simply be included in the "cystine" analysis. In the reaction between sulfite and cystine we find, however, that exactly 50 per cent of the cystine is reduced. If a protein hydrolysate contains cystine and cysteine, no error in analysis occurs because a cystine standard is used for color comparison. When, on the contrary, an hydrolysate contains cysteine and no cystine, an error of 100 per cent will be made, if a cystine standard is used. One way to avoid this error is to oxidize any cysteine present to cystine before treating the latter with sulfite. This can be done by denaturing the protein and oxidizing its SH groups with hydrogen peroxide before hydrolysis, for no SH groups are uncovered during hydrolysis. In this way the total cystine and

cysteine of a protein may be estimated in the form of cystine. If the denatured protein does not give a positive nitroprusside reaction, it contains no cysteine and preliminary oxidation is therefore not necessary. Most of the proteins analyzed by Folin and Marenzi contain no cysteine so that their cystine estimations of these proteins are correct. Even in the case of egg albumin which contains some cysteine their analysis is correct because drying of protein by their procedure may oxidize it. If, following the suggestion of Marenzi (1930), proteins are hydrolyzed without being dried, the estimation of cystine in egg albumin would be 50 per cent too high.

The evidence that only one-half of the cystine in a solution is reduced by sulfite will now be given. Folin and Looney observed that when cystine is treated with increasing amounts of sulfite, a point is reached at which a further increase in concentration of sulfite does not cause an increase in intensity of color when the reduced cystine is mixed with phosphotungstate. Under these conditions, they concluded, cystine is completely reduced. We compared the color produced with phosphotungstate by such a cystine preparation with that produced by an equivalent amount of pure cysteine and found that in the presence of sulfite the cystine forms a color exactly twice as intense as does the equivalent amount of cysteine. This result published in a preliminary communication (Mirsky and Anson, 1930) has been confirmed by Clarke (1932), and he has also shown that the portion of cystine not reduced by sulfite is oxidized. Clarke states that he has been able to confirm the observation that a more intense color is produced by cysteine than by an equivalent amount of cystine in a general way only, for he obtained somewhat less than 100 per cent difference in color intensity. His failure to obtain a difference of as much as 100 per cent may be due to impurities in his cysteine preparations. Cysteine is prepared by the reduction of cystine and completeness of reduction must be tested by titration.

The fact that only one-half the cystine in a protein hydrolysate is reduced by sulfite makes it possible to estimate the cysteine content of a protein by comparing the colors the hydrolysates of oxidized and non-oxidized protein produce when treated with sulfite and phosphotungstate. If the latter contains no cysteine the two colors should be of the same intensity; if it contains cysteine and no cystine the

color given should be twice that given by the oxidized form; and in the case of a protein containing both cystine and cysteine, the color should be between once and twice that given by the oxidized form.

A better way to estimate cysteine is to dispense with sulfite and so not include cystine in the analysis. This method has already been described as part of the procedure for estimating protein SH groups. Estimations of cysteine by the two methods agree for some proteins (egg albumin, and reduced serum albumin, for example) but disagree for the proteins of muscle and of the crystalline lens. For these proteins cysteine estimations made by reducing the hydrolysate with sulfite and then developing color with phosphotungstate in an alkaline solution are higher than those made without sulfite and at the neutral point. The estimates made in alkali must be erroneous for in some instances more cysteine appears to be present than can be accounted for by the cystine found in the same proteins after oxidation. Cysteine estimations made by developing a color with phosphotungstate at the neutral point appear to be reliable for in most proteins investigated the cystine content of completely reduced proteins is found to be equivalent to the cystine content of the oxidized proteins.

The cystine content of a protein is obtained by subtracting its cysteine content from the total cystine plus cysteine content of oxidized protein.

EXPERIMENTAL

I. The Denatured Protein Preparations Used

(*a*) *Serum Albumin.*—Denatured serum albumin is prepared in the form of a dry powder by the acid acetone procedure (Anson and Mirsky, 1931).

(*b*) *Egg Albumin.*—Heat-coagulated egg albumin is prepared by dissolving about 1 gm. of crystalline egg albumin, suspended in the ammonium sulfate solution with which it was crystallized, in 100 cc. water and heating with gentle stirring at 90°C. until all the protein is insoluble. The mixture is diluted with 125 cc. of water, centrifuged, the supernatant fluid discarded, and the precipitate rubbed into a fine paste by adding a little water. To the suspension are added 10 cc. of a 20 per cent sodium sulfate solution and enough water to make a volume of 225 cc. After stirring the suspension mechanically for 5 minutes, it is centrifuged and the supernatant fluid is discarded.

(*c*) *Edestin.*—1 gm. of the dried crystalline preparation made by Hoffmann-La Roche is dissolved in 200 cc. of 0.01 N HCl and then coagulated by adding

20 cc. of concentrated trichloracetic acid solution (trichloracetic acid dissolved in an equal weight of water). The mixture is centrifuged and the supernatant fluid is discarded.

(*d*) *Proteins of Halibut Muscle.*—5 gm. (wet weight) of minced frozen muscle are ground in a mortar with 50 cc. of 5 per cent trichloracetic acid. The mixture is diluted to 250 cc. with 5 per cent trichloracetic acid, stirred, and then centrifuged. The supernatant fluid is discarded.

(*e*) *Proteins of the Crystalline Lens.*—Six crystalline lenses, taken from the eyes of oxen, are ground in a mortar until they form a homogeneous jelly. 5 per cent trichloracetic acid is added gradually and with mixing until a volume of 225 cc. is reached. The suspension is centrifuged and the supernatant fluid is discarded.

II. Estimation of Cysteine Content of Protein Hydrolysate

(*a*) *Drying of Protein Preparations.*—A preparation is first freed of any contaminating substances that may be present by washing with trichloracetic acid. The protein in a 250 cc. centrifuge flask is mixed with 200 cc. water and to the mixture are added 20 cc. of concentrated trichloracetic acid (trichloracetic acid dissolved in an equal weight of water). After centrifuging, the supernatant is discarded, and the process is repeated as many times as may be needed. The trichloracetic acid precipitate is mixed with 200 cc. acetone, stirred mechanically for 5 minutes, and then 1 cc. concentrated HCl is added to the suspension. After centrifuging, the protein precipitate is stirred with 100 cc. acetone to which 5 or 6 drops of HCl are added. The acetone is decanted again, and much of what adheres to the protein is removed by suction while the flask is immersed in hot water. When the protein has reached the consistency of a thick dough, it is ground in a hot mortar until the odor of acetone has disappeared. The fine powder is now dried in an oven at 108° for 30 minutes, when a constant weight is reached.

(*b*) *Hydrolysis*—50 to 700 mg. of dried protein are transferred to a 50 cc. Kjeldahl flask. 5 cc. of 6 N H_2SO_4 and 1 cc. butyl alcohol are added. The flask is attached to a reflux condenser and placed on a sand bath where it is gently boiled for 15 hours. The condenser is then disconnected, the butyl alcohol is boiled off, the contents in the flask cooled under the tap, transferred to a 25 cc. volumetric flask, and brought to this volume by dilution with water. After filtration the cysteine content of the hydrolysate may be estimated. The use of a color filter in the colorimetric comparison obviates the necessity of decolorizing the hydrolysate. If insufficient protein is available, only 2 cc. 6 N H_2SO_4 need be used for hydrolysis, and after hydrolysis this is diluted to 10 cc.

(*c*) *Color Formation.*—The volume of hydrolysate used, from 1 to 5 cc., should contain about 0.5 mg. cysteine. If less than 5 cc. are used the volume is brought to 5 cc. by addition of 1 N H_2SO_4. To this are added 16 cc. of concentrated urea solution (urea dissolved in an equal weight of water), 4 cc. of 3.4 M K_2HPO_4 – KH_2PO_4 pH 6.8 buffer and finally, with mixing, 1 cc. phosphotungstic acid. After an interval of 5 minutes the intensity of the blue color is compared with a standard

in a colorimeter. The standard used is a blue glass, the blue of which need not closely match that of the solutions used, for a red filter is attached to the eye-piece of the colorimeter. The blue glass is calibrated by comparison with the blue color that a standard cysteine solution produces with phosphotungstate. Phosphotungstic acid is prepared according to the directions of Folin and Marenzi.

(*d*) *Preparation of a Cysteine Solution for Calibrating the Blue Glass Used in Colorimetric Comparisons.*—The cysteine content of the solution to be used is first estimated. Cysteine is treated with an excess of ferricyanide and the quantity of ferrocyanide formed is estimated colorimetrically as Prussian blue. 60 mg. of cysteine hydrochloride are dissolved in 100 cc. 0.01 N H_2SO_4. To 1 cc. are added 0.25 cc. 1 M K_2HPO_4 – KH_2PO_4 solution pH 7.2, 0.2 cc. 0.5 M $K_3Fe(CN)_6$ and the mixture is allowed to stand for 2 minutes. Then 18 cc. water, 0.5 cc. 1 N H_2SO_4, and 5 cc. of a solution containing ferric sulfate and gum ghatti. At the same time Prussian blue is formed from a known quantity of ferrocyanide. A solution of $K_4Fe(CN)_6$ in water is prepared containing 1.73 mg. per cc., an amount equivalent to 1.0 mg. cysteine. To 0.5 cc. of this solution are added 19 cc. water, 0.5 cc. 1 N H_2SO_4, and 5 cc. of ferric sulfate-gum ghatti solution. After standing for 2 minutes the colors of the Prussian blue solutions are compared with the same blue glass used in measuring the blue color produced in phosphotungstate by cysteine. To 2 cc. of the cysteine solution, the concentration of which is now known, are added 3 cc. water, 16 cc. concentrated urea solution, 4 cc. 3.4 M K_2HPO_4 – KH_2PO_4 pH 6.7 buffer and, with stirring, 1 cc. phosphotungstic acid. After standing for 5 minutes the color of this solution is compared with that of the blue glass which is to be used as a standard.

The fixity of the scale on the colorimeter should be verified occasionally. For this purpose the color of a solution of copper sulfate, containing 7 gm. in 100 cc. is compared with that of the blue glass.

III. Estimation of Cystine Content of Oxidized Proteins

A denatured protein is oxidized if it gives no color reaction with nitroprusside or if its hydrolysate does not give a blue color with phosphotungstate when tested as described in II (*c*). In the case of a protein, serum albumin for instance, that is oxidized in its natural state, no further oxidation is necessary.

(*a*) *Oxidation.*—To oxidize protein SH groups the denatured protein (in the case of egg albumin about 500 mg. are used) is mixed with enough 0.5 M pH 9.6 borate buffer to make the mixture definitely blue to thymol blue, and enough water is added to bring the volume to 100 cc. 5 cc. of 30 per cent H_2O_2 are added. After standing with occasional agitation for 30 minutes at room temperature, 100 cc. water and 25 cc. concentrated trichloracetic acid are added. The suspension is centrifuged, the supernatant decanted, and enough water is added to the precipitate so that with the aid of a rubber policeman it is rubbed first into a thick and then a thin paste containing the albumin in a very fine state of subdivision. Water is added to a volume of 200 cc., the mixture is stirred

mechanically from 5 to 10 minutes, and then 20 cc. of concentrated trichloracetic acid are added. The washing process is repeated four times.

(*b*) The protein is dried and hydrolyzed as described in II (*a*) and II (*b*).

(*c*) *Color Formation.*—The volume of hydrolysate used, from 1 to 5 cc., should contain about 0.7 mg. cystine. When less than 5 cc. of hydrolysate is used, enough 1 N H_2SO_4 is added to bring the volume to 5 cc. To this is added 1 cc. of a freshly prepared sodium sulfite solution, made by dissolving 2 gm. of the anhydrous powder in 10 cc. of water. After standing 1 minute, add 14 cc. concentrated urea solution, 4 cc. 3.4 M K_2HPO_4—KH_2PO_4 pH 6.7 buffer with stirring, 1 cc. phosphotungstic acid. Color intensity is measured, after an interval of 5 minutes as in II (*c*). The cystine solution for calibrating the blue glass used for color comparison is made by dissolving recrystallized cystine in 1 M H_2SO_4, 0.7 mg. per cc.

IV. The Cystine Content of Proteins That Are Not Completely Oxidized

This is obtained by subtracting the cysteine content of the unoxidized protein (II) from the cystine content of oxidized protein (III).

V. Protein SH Groups Estimated by the Direct Method

(*a*) *The Cystine Solution.*—To 350 mg. of recrystallized cystine are added 5 cc. of water and 6.75 cc. 0.5 N KOH. The cystine dissolves, and the solution is shaken in a tube which is evacuated by means of a vacuum pump. This solution must be made shortly before it is to be used, for cystine is unstable in alkaline solution.

(*b*) *The Reaction between Protein and Cystine*—Denatured protein (about 200 mg. in the case of egg albumin) is transferred to a 50 cc. centrifuge tube, mixed with 45 cc. of water and 2 cc. of concentrated trichloracetic acid. After centrifuging and decanting the supernatant, the protein is mixed with 15 cc. of a 20 per cent sodium sulfate solution and 30 cc. water, stirred and centrifuged. The precipitate is mixed with 5 cc. of sodium sulfate solution, and to this mixture is added the cystine solution. The tube is quickly stoppered, evacuated, filled with nitrogen (that is oxygen-free), evacuated again, again filled with nitrogen and allowed to stand at room temperature with occasional mixing for 30 minutes.

(*c*) *Estimation of Cysteine Formed.*—The protein is completely precipitated by adding 2 cc. of 10 per cent sodium tungstate and then 6 cc. $\frac{2}{3}$ N H_2SO_4, so that the mixture is red to litmus but not blue to Congo red. After centrifuging, the total volume of the contents of the tube is measured. A measured volume of the supernatant is then removed, mixed with 4 cc. of a 3.4 M KH_2PO_4 solution of pH 6.8, with 1 cc. 0.5 N KOH and with enough water to bring the volume to 25 cc. To this is added 1 cc. of either phosphomolybdic acid or phosphotungstic acid, and after 5 minutes the intensity of the blue color is measured in a colorimeter, as described in II (*c*). Phosphomolybdic acid is prepared (Wu, 1920) as follows: 100 gm. $Na_2MoO_4 + 2H_2O$ are dissolved in 450 cc. water and to the

solution are added 15 cc. of 85 per cent H_3PO_4 and 80 cc. of concentrated HCl. The solution is boiled for 8 hours under a reflux condenser and then, without allowing the solution to cool, a few drops of bromine are added to remove the blue color. The excess of bromine is driven off by boiling. The solution is now brought to a volume of 1000 cc. and is ready for use.

(*d*) *Estimation of Protein Concentration.*—The protein which has been treated with cystine is washed three or four times in a 50 cc. centrifuge tube with 5 per cent trichloracetic acid, separating the washings each time by centrifuging and decanting. The protein is then dissolved by adding 2 to 3 cc. of 0.5 N NaOH and enough concentrated urea solution (urea dissolved in an equal weight of water) to bring the volume of the solution to 25, 50, or 100 cc. For a protein such as reduced serum albumin, only a small quantity of protein is used, and the volume is accordingly brought to 25 cc., but when more protein is used, as in the case of egg albumin, the volume is adjusted to 100 cc.

VI. Estimation of Protein SH Groups by the Indirect Method

Either the procedure described below in (*a*), (*b*), and (*c*), or that in (*a'*), (*b'*), and (*c'*) can be followed.

(*a*) The cysteine content of a portion of the protein is estimated (II).

(*a'*) The SH groups of one portion of protein are estimated (V).

(*b*) A portion of the protein is either oxidized or treated with iodoacetate. Oxidation by H_2O_2 is described in III (*a*).

Before the protein is treated with iodoacetate, the trichloracetic acid precipitate of denatured protein (containing about 1 gm. of protein) is neutralized. The precipitate is stirred with a mixture containing 100 cc. 1 M K_2HPO_4–KH_2PO_4 solution of pH 7.6, 75 cc. of water, and 25 cc. saturated ammonium sulfate. After centrifuging the protein suspension and decanting the supernatant, the precipitate is mixed with a solution containing 60 cc. 0.5 M K_2HPO_4–KH_2PO_4 solution of pH 7.3 and 33 cc. 0.1 M iodoacetate (iodoacetic acid neutralized with phenol red as indicator). The mixture remains at room temperature with occasional agitation for 3 hours. To it are then added 100 cc. of water and 25 cc. of concentrated trichloracetic acid. The protein is now washed four times as described in III (*a*).

(*c*) The protein prepared in (*b*) is dried, hydrolyzed, and its cysteine content is estimated (II).

(*c'*) The SH groups of a protein prepared in (*b*) are estimated (V).

(*d*) The number of SH groups that reacted with H_2O_2 or with iodoacetate is calculated by subtracting the value found in (*c*) from that found in (*a*), or that in (*c'*) from that in (*a'*). For denatured proteins the values in (*c*) and (*c'*) are zero.

VII. Estimation of Protein S-S Groups

(*a*) The cysteine content of one portion of the protein is estimated (II), or

(*a'*) The SH groups of one portion of the protein are estimated (V).

(*b*) *Reduction of S-S Groups.*—

Serum Albumin.—200 mg. of denatured serum albumin are dissolved in 5 cc. water. 10 cc. of a saturated (at 30°C.) Na_2SO_4 solution are added slowly and with mixing so that a finely divided precipitate is obtained. 1 cc. of redistilled thioglycollic acid is neutralized with a 0.4 N KOH solution, containing saturated Na_2SO_4, so that the mixture is just red to phenol red, and then 0.5 cc. more alkali is added to compensate for the acidity of the albumin. The solution is diluted to 60 cc. with saturated Na_2SO_4, mixed with the albumin precipitate, and the mixture is allowed to stand in a 100 cc. stoppered flask with occasional stirring for 45 minutes at 30°C. At the end of this time the mixture is diluted with water to approximately 200 cc., and 20 cc. of concentrated trichloracetic acid solution are added with stirring. The washing process described in III (*a*) is repeated seven or eight times completely to free the albumin of thioglycollic acid.

Reduction is carried out in presence of concentrated Na_2SO_4 to keep the albumin precipitated and so prevent any reversal of denaturation that might otherwise occur. A temperature of 30°C. is needed to obtain sufficiently concentrated Na_2SO_4 solutions. Ammonium sulfate, which is sufficiently soluble at room temperature, cannot be used because it inhibits reduction.

Egg Albumin.—When denatured egg albumin is reduced about 500 mg. are used.

(*c*) The cysteine content of reduced protein is estimated (II), or

(*c'*) SH groups of reduced protein are estimated (V).

(*d*) The number of S–S groups is calculated by subtracting either (*a*) from (*c*), or (*a'*) from (*c'*).

RESULTS

Sufficient experimental results are now given to demonstrate the validity of the methods for a number of proteins. It should not be assumed, however, that the methods are applicable to all proteins, for in at least one case difficulties are encountered. Detailed studies to be published later, have been made of protein SH and S-S groups under various conditions. Estimations of protein SH and S-S groups are recorded in terms of cysteine and cystine. The methods yield results reproducible to within 10 per cent.

Egg Albumin

1. SH groups of denatured proteins estimated by reduction of cystine by the protein —0.56 per cent.

2. SH groups of *reduced* denatured protein by the reduction of cystine —1.13 per cent.

3. S-S groups of denatured egg albumin, that is (2) minus (1), —0.57 per cent.

4. Cysteine of untreated protein estimated by the reaction of the hydrolysate with phosphotungstate —0.616 per cent.

5. Cysteine of denatured egg albumin oxidized with hydrogen peroxide, estimated after hydrolysis by reaction with phosphotungstate —none.

6. SH groups of denatured protein by the indirect method, *i.e.* (4) minus (5), —0.616 per cent.

7. SH groups of *reduced* denatured protein by reaction of the hydrolysate with phosphotungstate —1.15 per cent.

8. S-S groups of denatured egg albumin by the indirect method, *i.e.* (7) minus (4), —0.534 per cent.

9. Cystine content of denatured egg albumin, oxidized with hydrogen peroxide, by the Folin-Marenzi procedure —1.24 per cent.

10. Uncorrected "cystine" content by the Folin-Marenzi method —1.93 per cent.

Serum Albumin

1. SH groups detectable either before or after hydrolysis —none.

2. SH groups of *reduced* denatured protein estimated by reduction of cystine, the direct method —4.57 per cent.

3. S-S groups of denatured protein calculated from (2), *i.e.* (2) minus (1), —4.57 per cent.

4. Cysteine of *reduced* denatured protein by reaction with phosphotungstate —4.73 per cent.

5. SH groups of *reduced* protein by the indirect method, *i.e.* (4) minus (1), —4.73 per cent.

6. Cystine content by the Folin-Marenzi method. No correction is needed, due to (1) —4.85 per cent.

Edestin

1. SH groups of protein denatured by trichloracetic acid and then reduced as estimated by the direct methods —1.18 per cent.

2. Cysteine content of reduced denatured protein by reaction of hydrolysate with phosphotungstate —1.20 per cent.

3. Cystine content of denatured protein oxidized with hydrogen peroxide —1.24 per cent.

Mixed Proteins of the Crystalline Lens

1. SH groups of denatured proteins estimated by the direct method —1.23 per cent.

2. Cysteine of the hydrolyzed proteins after the denatured, but unhydrolyzed, proteins were treated with iodoacetate —none.

3. Cysteine content of hydrolyzed, untreated proteins —1.24 per cent.

4. SH groups of denatured proteins by the indirect method, *i.e.* (3) minus (2), —1.24 per cent.

5. Cystine content of denatured proteins after oxidation by hydrogen peroxide —1.105 per cent.

6. Uncorrected cystine content of proteins by the Folin-Marenzi method—2.92 per cent.

Mixed Proteins of Halibut Muscle

1. Cysteine content of hydrolyzed proteins —0.807 per cent.

2. Cysteine content of hydrolysate after the denatured but unhydrolysed proteins were treated with iodoacetate —none.

3. SH groups of denatured proteins by the indirect method, *i.e.* (1) minus (2), —0.807 per cent.

4. Cystine content of denatured proteins oxidized by hydrogen peroxide —1.16 per cent.

5. Uncorrected "cystine" content by the Folin-Marenzi method —1.94 per cent.

6. Cystine content reported by Sullivan and Hess (1931) using the Sullivan method —1.13 per cent.

SUMMARY

1. Methods have been described for reducing protein S-S groups, for oxidizing protein SH groups, and for estimating protein S-S and SH groups.

2. It has been found necessary in estimating the cystine content of proteins by the Folin-Marenzi method to take into account any cysteine that may be present.

3. A method for estimating the cysteine content of proteins has been described.

4. With these methods, estimations have been made of the S-S and

SH groups and of the cystine and cysteine contents of a number of proteins.

5. In a denatured, but unhydrolyzed protein, the number of S-S and SH groups is equivalent to the quantity of cystine and cysteine found in the protein after hydrolysis.

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