SULFHYDRYL AND DISULFIDE GROUPS OF PROTEINS

III. Sulfhydryl Groups of Native Proteins—Hemoglobin and the Proteins of the Crystalline Lens

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In a denatured but unhydrolyzed protein the number of SH and S-S groups detectable is equivalent to the quantity of cysteine and cystine found in the hydrolyzed protein (Mirsky and Anson, 1934–35).[1] Detectable protein SH groups react with iodoacetate or with oxidizing agents such as ferricyanide and cystine. Detectable protein S-S groups have the property of being reduced to SH groups by a thiol compound such as thioglycollic acid. The methods of detection of these groups are applicable without any modifications to native as well as denatured proteins. When these methods are applied to native proteins which are under the same conditions as are the denatured proteins, some (egg albumin, for instance) manifest no groups at all, while others (serum albumin) show only a fraction of the number present in the denatured form.

Since the typical protein, egg albumin, has no detectable SH groups, it might be suspected that the S-S groups detected in supposedly native serum albumin (Mirsky and Anson, 1935–36) are present only because the procedure for detecting groups has caused some denaturation. In the present investigation, however, SH groups are demonstrated in native hemoglobin, and in this case there is some assurance that the procedure does not cause denaturation, for denatured hemoglobin can readily be detected spectroscopically (Anson and Mirsky, 1925, 1928–29). The disadvantage of using hemoglobin, that heme interferes with estimation of SH groups, can be avoided.

[1] This paper will be referred to as (Paper I).

A study of the SH groups of native hemoglobin shows that the number of groups detectable is dependent on hydrogen ion concentration, the number increasing as the pH rises. At pH 6.8 no groups are detectable but as the pH is raised (in our experiments as far as 9.5) more and more groups appear. When the pH is brought back to 6.8 they are no longer detectable.

These observations raise the question as to how the SH groups of native hemoglobin differ from those of denatured hemoglobin. The difference between the groups of native and denatured proteins is that the latter can be detected at a pH at which the former cannot be. It is found that at pH 6.8, where no groups are detectable in native hemoglobin, SH groups are detectable in denatured globin; and the number detectable is the maximum available; that is, it is equal to the number of cysteine molecules found in hydrolyzed globin. In comparing native hemoglobin with denatured globin, the differences observed may in part be due to the presence or absence of heme as well as to the state of the protein. The same differences, however, are observed between the native and denatured forms of other proteins, the proteins of the crystalline lens, for instance. In general, then, the effect of denaturation is to extend towards the acid side the pH range in which SH groups are detectable. In every thiol compound (thioglycollic acid, cysteine, and glutathione, for example) activity of the SH group in its reactions with oxidizing agents or with iodoacetate increases with a rise in pH, and in this respect both native and denatured proteins resemble other thiol compounds. But even without change in pH, the SH groups of a protein can be activated by denaturation.

The experiments on hemoglobin and the lens proteins show that activation of SH groups can serve as a satisfactory criterion of denaturation. The test for SH groups activated by denaturation should be carried out at a pH so low that the groups of native protein are inactive and yet high enough for those of denatured protein to be active. Proper conditions for the test vary accordingly from one protein to another, for each protein has its characteristic curve relating pH to activity of groups. The SH groups of the native lens proteins become active at a pH below 6.8, those of hemoglobin above pH 7.0, and those of egg albumin are inactive even at pH 9.6.

SH Groups of Hemoglobin

The method used for estimating the SH groups of hemoglobin is substantially the same as the "indirect" method of Mirsky and Anson (Paper I). Hemoglobin at the desired pH is treated with potassium ferricyanide[2] to oxidize any SH groups present, and the excess ferricyanide is removed by dialysis. Globin and heme are then separated by the acid-acetone procedure (Anson and Mirsky, 1929–30) so that the heme will not interfere with the subsequent analytical procedure for oxidized heme reacts with thiol compounds. The SH groups of the denatured globin prepared in this manner are estimated and compared with the number found in globin prepared from hemoglobin which was not treated with ferricyanide. The difference between these values is equal to the number of SH groups oxidized by ferricyanide, and it is accordingly a measure of the number of SH groups present in hemoglobin under the conditions of the experiment.

The number of SH groups found in native hemoglobin is dependent upon the hydrogen ion concentration. At pH 6.8 almost no groups are detectable; in a pH 7.3 buffer, 28 per cent of the total number of groups contained in the protein appear; in pH 9.0, 44 per cent; and in pH 9.5, 65 per cent are found. The effect of change in pH is reversible. If the pH is brought to 8.75 and then, after an interval to 6.8, no groups are detectable at the latter pH.

The experiments at different hydrogen ion concentrations show that the iron porphyrin part of the hemoglobin molecule can be oxidized independently of its SH groups, for at pH 6.8 ferricyanide oxidizes all of the hemoglobin to methemoglobin without oxidizing any of the SH groups. And, conversely, it is possible to oxidize SH groups of hemoglobin without oxidizing other parts of the molecule. This can be done at pH 9.6 by using cystine, a very mild oxidant, to oxidize SH groups without simultaneous formation of methemoglobin.

When the SH groups of hemoglobin are oxidized at pH 9.5, either by ferricyanide or cystine, no denatured protein is present by spectroscopic test (Anson and Mirsky, 1925, 1928–29). If ferricyanide is

[2] The potential of the ferrocyanide-ferricyanide system is constant in the pH range used in these experiments.

used, methemoglobin is formed, and if cystine is used, the hemoglobin remains unchanged spectroscopically; in neither case is any parahematin or hemochromogen, that is denatured hemoglobin, observed spectroscopically.

SH Groups of the Proteins of the Crystalline Lens

The lens proteins are treated with iodoacetate which destroys active SH groups and the excess iodoacetate is removed simply by precipitating the protein with trichloracetic acid and washing. In this

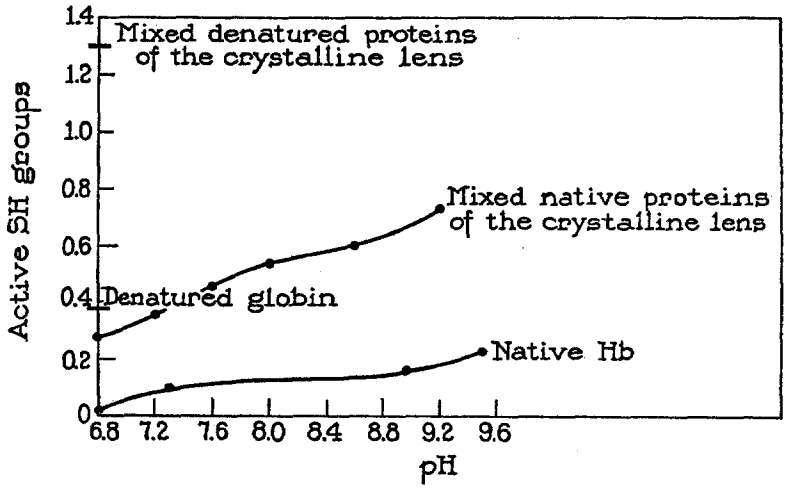

FIG. 1. Relation between pH and number of active SH groups in hemoglobin and in the mixed proteins of the crystalline lens

process the protein is denatured, but in presence of acid the SH groups of denatured proteins are inactive. The number of groups that react with iodoacetate is found by hydrolyzing the protein and estimating its cysteine content. The difference between the cysteine contents of proteins treated with iodoacetate and of those not treated is a measure of the number of SH groups that react with iodoacetate. When these proteins are said to be in the native state, it is meant that in their preparation no agent known to denature proteins is employed. Evidence, such as that available for hemoglobin preparations, that no denatured protein is present, is lacking. And yet a study of the

activity of SH groups indicates that there is little if any, denatured protein in preparations of the lens proteins, for behavior of the groups is similar to that of the SH groups of native hemoglobin. Activity of the SH groups of the native lens proteins is augmented as the pH rises. The effect is reversible; groups activated by a rise in pH lose their activity when the pH drops. When these proteins are denatured, their SH groups become fully active at a pH at which groups of the native proteins are only just beginning to be active.

EXPERIMENTAL

The SH groups of globin were first detected by means of the nitroprusside reaction (Anson and Mirsky, 1930–31). It was then recognized by Schüler (1932) that in the reaction between hemoglobin and ferricyanide these groups, as well as heme, might react with ferricyanide. To estimate the number of SH groups of hemoglobin that might take part in this reaction Schüler titrated globin with ferricyanide. It was assumed that ferricyanide would react only with the SH groups of globin. His experiments have been repeated and his observations confirmed; furthermore, his estimate of the number of SH groups in globin (of the guinea pig) is the same as those we have made (of horse globin) by entirely different methods which appear to be specific for SH groups. When, however, other proteins are treated with ferricyanide we find that there are reducing groups in addition to the SH which react with ferricyanide. These hitherto unrecognized reducing groups of proteins will be described in another paper. Another assumption made by Schüler was that in globin and hemoglobin the same number of SH groups react with ferricyanide. It is shown in this paper that only part of the SH groups of globin are active in hemoglobin. The number active increases as the pH rises. The behavior of the SH groups of hemoglobin should not be neglected in studies on the oxidation-reduction potential of the hemoglobin-methemoglobin system.

It has been claimed by Meldrum (1932) that neither SH nor S-S groups are detectable in globin, and that the observations of Anson and Mirsky can be explained by a failure to distinguish between the color reaction given by nitroprusside with acetone, and that given with SH groups. The evidence for the existence of SH and S-S groups in globin may be briefly summarized:

1. Horse globin prepared by the acid-acetone procedure and then thoroughly washed with trichloracetic acid to remove acetone gives a marked color reaction with nitroprusside and ammonium hydroxide. This color is distinctly different from the color given by acetone. Serum albumin prepared by the acid-acetone procedure and then washed free of acetone does not give a color reaction with nitroprusside and ammonium hydroxide; neither does it contain SH groups, when tested by other methods. This color reaction of horse globin is unmistakable. Only if insufficient care is taken in preparing globin, in which case a deeply pig-

mented protein instead of a colorless one is obtained, are these reactions obscure, as stated by Meldrum.

Globin was treated in the following manner before being tested with nitroprusside; about 100 mg. of protein, with acetone still adhering to it, were mixed with a little water, so as to form first a thick and then a thin paste. About 200 cc. of water were then added and the mixture stirred mechanically for 15 minutes, when 20 cc. of a 50 per cent solution of trichloracetic acid were added. After centrifuging the supernatant fluid was discarded, and the protein was washed again in the same manner. This process was repeated four times. The protein was then washed with sodium sulfate and a pH 7.3 KH_2PO_4-K_2HPO_4 buffer as described below (p. 447). After centrifuging, a little of the protein (now in the form of a very thick paste) was placed on a piece of folded filter paper. The protein was moistened with a few drops of a 5 per cent solution of sodium nitroprusside, the filter paper absorbing the excess fluid. The protein was now moistened with a few drops of dilute ammonium hydroxide.

2. Denatured horse globin reduces cystine to cysteine. This reaction, which is certainly not due to acetone, depends on the presence of SH groups in the protein. The experimental procedure for this reaction is described below.

3. After horse globin has been oxidized with ferricyanide, as described below, it gives no test with nitroprusside for SH groups but it now gives an intense test for S-S groups. Globin treated with ferricyanide does not reduce cystine. To test for S-S groups a few small crystals of potassium cyanide were placed on the protein (on a piece of filter paper, as in the test for SH groups) before adding nitroprusside. No ammonium hydroxide was used (Walker, 1925).

4. When hydrolyzed horse globin reacts with phosphotungstate a blue color is formed indicating the presence of cysteine. Globin oxidized by ferricyanide and then hydrolyzed contains no cysteine. This indicates that before oxidation globin contained a number of SH groups equivalent to the cysteine content of non-oxidized hydrolyzed globin (Paper I).

5. If horse globin is dried in an oven at 110°, in the course of many days its SH groups, as tested for by nitroprusside, gradually disappear; the test for S-S groups does not become negative.

6. Ox globin was prepared by Wu's method, which does not involve the use of acetone, but in which there is ample opportunity for oxidation of SH groups. This globin, when treated with nitroprusside, gives no test for SH groups but does give an intense test for S-S groups.

7. If ox globin, prepared by Wu's method, is treated with acetone which is then washed away, the protein does not now appear to contain SH groups due to traces of acetone which, it may be imagined, were not removed. It gives no color reaction with nitroprusside and ammonium hydroxide, although the protein, due to the treatment with acetone, is so white that even a faint color could be detected. This protein gives an intense test for S-S groups.

Haurowitz (1935) believes that globin contains SH groups, although he was unable to obtain a test with nitroprusside using either ammonium hydroxide or

potassium cyanide. He has suggested that it may be through its thiol groups that globin is attached to heme. This theory, for which Haurowitz advanced no convincing evidence whatsoever, is untenable for several reasons:

1. More than half of the SH groups of globin can be oxidized while the globin is joined to heme in the form of hemoglobin without disrupting the molecule.

2. A molecule of globin of the horse contains only two SH groups, although it can combine with four heme molecules.

Vickery and White (1933) have devised a method for estimating the cystine content of proteins that is entirely different from the method used in our investigations. In their method no distinction is made between cystine and cysteine: quantities of both are lumped together as "cystine" content. Their estimate of the "cystine" content of horse hemoglobin (0.41 per cent) is in good agreement with our value for the *cysteine* content of horse globin (0.42 per cent). This agreement indicates that horse globin contains cysteine but not cystine. Our estimate of cystine content, however, does not agree with that reported by Vickery and White. Since horse globin contains cysteine it is to be expected (Mirsky and Anson, 1930) that an estimation of its cystine content by the Folin-Marenzi (1929) method would be too high, for Folin and Marenzi assume that cysteine and cystine have the same color value with their reagent, whereas cysteine actually gives twice as much color as cystine. (The estimate is 1.14 per cent.) But even after all of the cysteine of globin has been oxidized to cystine, its cystine content, as given by a modification (Paper I) of the Folin-Marenzi method, is 0.63 per cent, distinctly higher than the value reported by Vickery and White.

Cystine may not be the only substance in a hydrolysate of oxidized globin that reacts with the phosphotungstate of Folin and Marenzi in presence of sulfite. That in the hydrolysates of a number of proteins no other substance is present, is shown by the evidence presented in a previous paper (Paper I) and also by the agreement in estimates of cystine content of several proteins by the Folin-Marenzi and the Vickery-White methods. And yet there is evidence that under certain conditions protein hydrolysates do contain interfering substances. We find, using the Folin-Marenzi method, that the cystine content of serum globulin is 2.2 per cent, which is in fair agreement with the value (1.82 per cent) obtained by the Sullivan method.[3] In hydrolyzing the protein 6N H_2SO_4 was used. If more concentrated acid is used, the estimate of cystine content, using the Folin-Marenzi reagent rises to 3.0 per cent if 10N H_2SO_4 is used and to 3.4 per cent if 11.3N H_2SO_4 is used.[4] These results suggest that when globin is hydrolyzed

[3] Our thanks are due to Dr. Sullivan for the analysis by his method (1926).

[4] These experiments were carried out because of the estimates of cystine content of serum globulin reported by Tuchman and Reiner and by Reiner and Sobotka, using a modification of the Folin-Marenzi method. Their estimates, ranging from 2.34 per cent to 4.70 per cent and averaging 3.64 per cent are distinctly higher and more variable than ours, made with the unmodified Folin-Marenzi method. Their modification was to precipitate the protein with trichlor-

with 6N H_2SO_4 substances interfering with the estimation of cystine by the Folin-Marenzi method may possibly be formed.

It was thought that an estimate of the number of S-S groups in denatured globin by reducing them with thioglycollic acid and estimating the SH groups formed, might serve as a check on the cystine estimation because in some denatured proteins the number of S-S groups is equivalent to the cystine content. Unfortunately estimations of the S-S groups of denatured globin yield results that are both so variable and so high (over 1 per cent) that it appears doubtful whether the method of estimation is applicable to globin. It is possible that thioglycollic acid remains adsorbed to the protein. In the absence of confirmatory evidence it is unlikely that estimation of the cystine content of globin by our method is correct.

Finally, it should be stated that the difficulties encountered in estimating the cystine content of globin do not affect the results reported in this paper, for confidence can be placed in our estimate of the cysteine content of globin. In this case estimation of SH groups serves as a check, since it is found that the number of SH groups in denatured globin is equivalent to the cysteine content of hydrolyzed globin.

Hemoglobin. Reaction between Hemoglobin and Ferricyanide

The reagents used were a 10 per cent solution of horse oxyhemoglobin prepared by Heidelberger's method, M/2 potassium ferricyanide, 3.4 M KH_2PO_4-K_2HPO_4 pH 6.8, M/2 KH_2PO_4-K_2HPO_4 pH 7.3, M/1 K_2HPO_4, M/2 H_3BO_3-NaOH pH 9.0, and M/2 H_3BO_3-NaOH pH 9.6. 5 cc. hemoglobin solution were mixed with 10 cc. buffer and 3 cc. ferricyanide, and the mixture allowed to stand at room temperature for 30 minutes. Under these conditions all the SH groups of denatured globin react with ferricyanide. 8 cc. of the pH 6.8 buffer were then added, and the solution was dialyzed against distilled water in a rocking dialyser for 20 hours to remove the ferricyanide. After adding the various buffer solutions to hemoglobin the pH of the resulting mixtures was measured with the glass electrode, with the following results: after adding the pH 7.30 buffer, the pH of the mixture was 7.30; after adding K_2HPO_4 the pH was 8.75; after adding the pH 9.00 buffer, the pH was 8.94; and after adding the pH 9.6 buffer, the pH was 9.5.

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acetic acid and hydrolyze the precipitated protein with 14N H_2SO_4 instead of drying the protein and hydrolyzing with 6N H_2SO_4. Following their procedure we obtained, in agreement with them, a cystine content of 3.44 per cent. The final concentration of H_2SO_4 in the hydrolysate was about 11.3N. The modification of the Folin-Marenzi method introduced by Tuchman, Reiner, and Sobotka accounts for the high values they obtained, and it probably also accounts for the variability of their results, for it is unlikely that the concentration of H_2SO_4 used by them for hydrolysis was kept constant.

Reaction between Hemoglobin and Cystine

A concentrated cystine solution was prepared by adding to 0.75 gm. cystine N/2 KOH (about 12.5 cc.) until practically all the cystine dissolved, but not enough alkali to make the pH exceed 9.6. The solution was blue to thymol blue but colorless to thymolphthalein. To this were added 5 cc. of a 10 per cent solution of horse carbon monoxide hemoglobin. Carbon monoxide was bubbled through the solution, the flask was then stoppered and allowed to stand in the dark for 1½ hours when 10 cc. of 3.4 M pH 6.8 K_2HPO_4-KH_2PO_4 buffer were added. The precipitated cystine was removed by centrifuging.

Preparation of Globin. The acid-acetone method was used to prepare globin from hemoglobin that had been treated with ferricyanide or cystine. In preparing globin from hemoglobin that had been treated with cystine, it was necessary to add more acid than is usually employed because of the phosphate buffer present. To the hemoglobin solution were added 10 cc. N HCl and to the 600 cc. of acetone used another 10 cc. N HCl were added. Removal of heme made it possible to estimate the SH groups of hemoglobin by the methods used for other proteins. The globin precipitated by acetone was not separated by filtration but by centrifuging. This was done in a 250 cc. centrifuge flask, and the globin was washed free of pigment by further additions of acid-acetone. Most of the acetone was removed by centrifuging, and the rest was removed by washing several times with 5 per cent trichloracetic acid.

Estimation of SH Groups of Untreated and Oxidized Globin.—The SH groups of the various preparations of globin were estimated by the "direct" method (Paper I). Globin was mixed with a cystine solution and the quantity of cysteine formed was equivalent to the number of SH groups of the protein. The number of active SH groups of any given sample of hemoglobin was equal to the difference between the number of SH groups in globin prepared from untreated hemoglobin and the number in globin prepared from hemoglobin that had reacted with ferricyanide or cystine.

Reversal of pH Effect.—To 5 cc. of the hemoglobin solution were added 10 cc. M K_2HPO_4 and after 30 minutes 6 cc. 3.4 M K_2HPO_4-KH_2PO_4 pH 6.8. This globin was treated with ferricyanide and then dialyzed as described above.

The cysteine content (SH groups of hydrolyzed globin) was estimated by the method described by Mirsky and Anson (Paper I).

Reaction of Denatured Globin with Ferricyanide

0.5 gm. of denatured globin in the form of a dry powder prepared by the acid-acetone method (Anson and Mirsky, 1929–30) was dissolved in 50 cc. of water. This was diluted with water to a volume of 200 cc. and to the solution were added 15 cc. of concentrated trichloracetic acid (trichloracetic acid dissolved in an equal weight of water). The mixture was centrifuged, the supernatant fluid discarded, and the precipitate transferred to a 50 cc. centrifuge tube. In this it was well stirred with 40 cc. of a 20 per cent sodium sulfate solution and 10 cc. of a 1.2

M pH 7.3 KH_2PO_4-K_2HPO_4 buffer. The suspension was centrifuged and the precipitate was mixed with 40 cc. sodium sulfate solution and 5 cc. 3.4 M KH_2PO_4-K_2HPO_4 pH 6.8 buffer. After centrifuging, the protein was suspended in a mixture of 25 cc. sodium sulfate solution, 3 cc. pH 6.8 buffer, and 4 cc. M/2 potassium ferricyanide. This tube stood for an hour with occasional agitation and was then centrifuged. The globin was washed with a 10 per cent solution of sodium sulfate by repeated mixing and centrifuging until no ferricyanide could be seen in the washings. The SH groups of this protein were estimated by the direct method, by the quantity of cysteine formed when the protein was mixed with a cystine solution (Paper I). Another sample was hydrolyzed, and the cysteine content of the hydrolysate estimated.

Experiments with the Lens Proteins

Lenses dissected from eyes of oxen were thoroughly mashed in a mortar. During the mashing small amounts of physiological saline were added so that a thin, homogeneous, paste-like mixture of the proteins was prepared. One portion was denatured with trichloracetic acid and then treated with iodoacetate to estimate the SH groups of the denatured proteins (Paper I). Other portions, while in the native state, were mixed with buffers varying in pH from 6.8 to 9.2 and were then treated with iodoacetate. The buffers, all M/2, were phosphate at 6.8, 7.2, 7.6, and 8.0 and borate at pH 8.6 and 9.2. To approximately 7 cc. of a protein mixture (containing about 600 mg. of protein) were added 66 cc. buffer solution and 33 cc. M/10 iodoacetate (iodoacetic acid neutralized with sodium hydroxide). From this point the procedure was the same as that of the "indirect" method for estimating protein SH groups (Paper I). The quantity of cysteine found in the hydrolysate of such a preparation was equivalent to the number of SH groups of the native protein that were *not* active at a given pH. By subtracting the number of inactive groups from the total number present, the number of active groups was obtained.

EXPERIMENTAL RESULTS

SH groups are recorded in terms of cysteine, that is as the quantity of cysteine which would have the same sulfur content, the amount of cysteine being expressed as per cent of the total amount of protein.

Globin and Hemoglobin

1. Cysteine content of hydrolyzed horse globin—0.42 per cent. This is equivalent to 2 molecules of cysteine per molecule of hemoglobin containing 4 iron atoms.

2. SH groups of denatured globin—0.38 per cent.

3. Cysteine content of hydrolyzed globin after denatured globin had been treated with potassium ferricyanide at pH 6.8—Nil.

4. SH groups of denatured globin prepared from hemoglobin treated with potassium ferricyanide at

(*a*) pH 6.8 —0.36 per cent
(*b*) pH 7.3 —0.28 per cent
(*c*) pH 8.96—0.22 per cent
(*d*) pH 9.5 —0.15 per cent

5. SH groups of native hemoglobin at

(*a*) pH 6.8 —(2) minus (4*a*)—0.02 per cent
(*b*) pH 7.3 —(2) minus (4*b*)—0.10 per cent
(*c*) pH 8.96—(2) minus (4*c*)—0.16 per cent
(*d*) pH 9.5 —(2) minus (4*d*)—0.23 per cent

6. SH groups of globin prepared from hemoglobin brought to pH 8.75 for 15 minutes and then treated with potassium ferricyanide at pH 6.8—0.35 per cent.

7. SH groups of globin prepared from carbon monoxide hemoglobin treated with cystine at approximately pH 9.6 (without methemoglobin formation in contrast to the experiments with ferricyanide)—0.14 per cent.

Proteins of the Crystalline Lens

1. Cysteine content of the protein hydrolysate—1.25 per cent.

2. Cysteine content of the protein hydrolysate after denatured protein had been treated with iodoacetate at pH 7.0—Nil.

3. SH groups of denatured protein (1) minus (2)—1.25 per cent.

4. Cysteine content of protein hydrolysate after the native protein had been treated with iodoacetate at

(*a*) pH 6.8—0.97 per cent
(*b*) pH 7.2—0.89 per cent
(*c*) pH 7.6—0.79 per cent
(*d*) pH 8.0—0.71 per cent
(*e*) pH 8.6—0.65 per cent
(*f*) pH 9.2—0.52 per cent

5. Active SH groups of native protein at

(*a*) pH 6.8 (3) minus (4*a*)	—0.28 per cent
(*b*) pH 7.2	0.36 per cent
(*c*) pH 7.6	0.46 per cent
(*d*) pH 8.0	0.54 per cent
(*e*) pH 8.6	0.60 per cent
(*f*) pH 9.2	0.73 per cent

6. Native proteins having been at pH 9.4 for 2 hours in the absence of oxygen treated with iodoacetate at pH 7.2—cysteine content of protein hydrolysate—0.90 per cent.

SUMMARY

Hemoglobin and the proteins of the crystalline lens contain active SH groups while in the native state, the number of active groups increasing as the pH rises. All the SH groups of denatured globin and of the denatured lens proteins are active at a pH so low that practically none of the SH groups of native hemoglobin and of native lens protein are active. The effect of denaturation on the SH groups of a protein is to extend towards the acid side the pH range of their activity.

It is possible to oxidize the iron-porphyrin and the SH groups of hemoglobin independently of each other.

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