SOME FACTORS WHICH INFLUENCE THE OXIDATION OF SULFHYDRYL GROUPS

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(Received for publication, August 6, 1941)

INTRODUCTION

The experiments described in this paper show (1) that the oxidation of SH groups by common oxidizing agents such as ferricyanide and Folin's uric acid reagent is inhibited by cyanide and promoted by copper sulfate, (2) that the SH groups of denatured egg albumin can be oxidized by an equivalent amount of ferricyanide even in the absence of denaturing agents, provided aggregation is avoided, (3) that the SH groups of denatured egg albumin are more easily oxidized by some oxidizing agents in urea or guanidine hydrochloride solution than in a solution of long chain alkyl sulfates1 or in the absence of denaturing agents, and (4) that the SH groups of egg albumin partially hydrolyzed by pepsin are more easily oxidized than the SH groups of denatured but unhydrolyzed egg albumin. It will be shown in another paper that urea and partial hydrolysis promote the oxidation of protein tyrosine and tryptophane groups as well as protein SH groups and that urea promotes the oxidation even of free tyrosine and tryptophane.

Now that it is clear that the oxidation of SH groups even by common oxidizing agents other than oxygen is dependent on cyanide-sensitive catalysts, the possibility must always be considered that a difference in the ease with which two SH compounds are oxidized may be due, in part at least, to a difference in the catalytic impurities present.

All denaturing procedures bring about the same qualitative changes in a protein's properties. The differences in the ease with which the SH groups of denatured egg albumin are oxidized in the presence of different denaturing agents such alkyl sulfate, urea, and guanidine hydrochloride, however, show

1 In a previous note (Anson, 1939a) it was stated that urea and guanidine hydrochloride, like Duponol PC, promote the reaction between ferricyanide and the SH groups of denatured egg albumin but that urea and guanidine hydrochloride are less effective than Duponol PC. By this statement was meant merely that the amount of Duponol PC needed to denature egg albumin and thus to make all the SH groups of denatured egg albumin react with dilute, neutral ferricyanide is much less than the amounts of urea or guanidine hydrochloride needed to achieve the same result.
that the exact reactions of a denatured protein depend on what denaturing agent is present. This dependence has also been shown with the nitroprusside test. The SH groups of denatured egg albumin give a strong pink color with nitroprusside in guanidine hydrochloride solution, a weaker color in urea solution, and a still weaker color in alkyl sulfate solution (Anson, 1941).

It is known from earlier experiments that alkyl sulfate, urea, and guanidine hydrochloride all can denature egg albumin, keep the denatured protein in solution, and make its SH groups accessible to titration. These earlier experiments, however, unlike the present experiments, were carried out in such a way that they did not permit any conclusions about differences in the ease of oxidation of the SH groups of denatured egg albumin in the presence of different denaturing agents. In the earlier experiments the SH groups of the denatured egg albumin were oxidized in neutral solution by relatively strong oxidizing agents such as porphyrindin and ferricyanide. Under such favorable conditions for oxidation the SH groups of denatured egg albumin are oxidized in alkyl sulfate solution as well as in urea solution. They are oxidized, as will be shown, even in the complete absence of substances such as urea and alkyl sulfate provided the denatured protein is prepared with proper precautions. In the present experiments, however, the conditions for the oxidation of the SH groups of denatured egg albumin are made more unfavorable by making the solution more acid or by using a weaker oxidizing agent, the uric acid reagent. Under the new, more unfavorable conditions oxidation of the denatured protein does not take place in the absence of denaturing agents or even in the presence of long chain alkyl sulfates. When urea or guanidine hydrochloride is added or when the protein is digested then oxidation takes place even under the less favorable conditions, and the effects of urea, guanidine hydrochloride, and hydrolysis on denatured egg albumin are thus readily demonstrated. Similarly the effect of added copper sulfate on the oxidation of cysteine by the uric acid reagent is demonstrated by carrying out the oxidation in an acid solution in which no oxidation takes place in the absence of added copper sulfate. In neutral solution, oxidation takes place without added copper sulfate.

The experiments described in this paper have helped to make clearer the factors which influence the oxidation of protein SH groups and they have suggested experiments with other protein groups. By themselves, however, the present experiments are not adequate to decide by what structural mechanisms aggregation, denaturing agents, and hydrolysis influence the SH reactions of egg albumin. Furthermore, although the urea effects which have been observed cannot be imitated by small amounts of copper sulfate, the present experiments do not decide to exactly what extent catalytic impurities contribute to the effects of various reagents on protein SH reactions. Finally, it is not known to what extent the uric acid reagent influences the surface structure and aggregation of denatured egg albumin.
EXPERIMENTAL RESULTS

Cyanide.—The oxidation of the SH groups of cysteine (Mathews and Walker, 1909) and denatured egg albumin (Rosenthal and Voegtlin, 1933) by oxygen is promoted by heavy metal compounds, especially copper salts, and indeed does not take place at all in the absence of heavy metal catalysts (Warburg and Sakuma, 1923). The work on the heavy metal catalysis of oxidation of SH by oxygen has recently been reviewed by Bemheim and Bemheim (1939).

Most commercial samples of guanidine hydrochloride contain impurities which catalyze the oxidation of the SH groups of denatured egg albumin by oxygen. This oxidation can be inhibited by cyanide (Anson, 1941). When the attempt was made to titrate the SH groups of denatured egg albumin in guanidine hydrochloride solution with ferricyanide in the presence of cyanide, added to prevent oxidation by oxygen, it was found that the added cyanide inhibited not only the oxidation of SH by oxygen but also stopped much of the oxidation of SH by ferricyanide. Cyanide also slowed up but did not prevent the oxidation of cysteine by ferricyanide (Anson, 1941). This inhibition of ferricyanide oxidation by cyanide indicated that even the ferricyanide oxidation requires heavy metal catalysts and led to the present experiments with cyanide and copper sulfate.

The oxidations of SH by the uric acid reagent described in this paper are more completely inhibited by cyanide than the ferricyanide oxidations previously studied. 1 drop of 0.1 N K CN in 10 cc. solution inhibits completely the oxidation of cysteine by neutral uric acid reagent. Somewhat more cyanide is needed to inhibit the oxidation of the SH groups of denatured egg albumin in neutral urea solution (Table I).

Heavy Metals.—Copper sulfate promotes the oxidation of cysteine by the uric acid reagent and ferricyanide.

The oxidation of cysteine by the uric acid reagent which takes place in alkaline solution (Folin and Looney, 1922) and in neutral solution (Lugg, 1932; Mirsky and Anson, 1935) does not take place at pH 4.8. If copper sulfate is added, however, the oxidation takes place even at pH 4.8 (Table I).

Similarly, in neutral solution 1 cc. of 0.001 M ferricyanide is reduced by 1 cc. of 0.001 M cysteine with disappearance of the brown color of ferricyanide. At pH 4.8 the brown color does not disappear. If copper sulfate is added at pH 4.8, however, the brown color of ferricyanide is replaced by the weak red color of copper ferrocyanide.

How acid the solution of cysteine has to be made to prevent oxidation by the uric acid reagent or ferricyanide varies with different samples of cysteine. Presumably different samples of cysteine contain different amounts of catalytic impurities. The sample of cysteine used for the experiments in Table I was not the most easily oxidized or the least easily oxidized of the samples of cysteine I have encountered. If the reagents were completely free of heavy metal impurities, oxidation would probably not take place even in neutral solution and even a trace of added copper sulfate would promote oxidation.
In comparing the oxidation of cysteine under different conditions it is important to keep the volume of solution in which the reaction takes place constant. At pH 5.2 cysteine is oxidized by the uric acid reagent 15 per cent if the reaction is carried out for 10 minutes in 10 cc. of solution. If, however, the reaction is carried out in 3 cc. with the same absolute amounts of reactants and the solution is diluted to 10 cc. at the end of 10 minutes, then 71 per cent of the cysteine is oxidized. In 6 cc. of reaction solution, the oxidation is 30 per cent complete.

### TABLE I

**Effect of CN and CuSO₄ on Oxidation of SH Groups**

<table>
<thead>
<tr>
<th>SH compound</th>
<th>pH</th>
<th>CuSO₄ ( \times 10^{-3} )</th>
<th>KCN ( \times 10^{-5} )</th>
<th>Oxidation per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>6.6</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Cysteine</td>
<td>6.6</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Albumin</td>
<td>6.9</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Albumin</td>
<td>6.9</td>
<td>0</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Albumin</td>
<td>6.9</td>
<td>0</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4.8</td>
<td>4</td>
<td>0</td>
<td>88</td>
</tr>
<tr>
<td>Cysteine</td>
<td>4.8</td>
<td>1</td>
<td>0</td>
<td>46</td>
</tr>
</tbody>
</table>

**Denaturing Agents.**—The SH groups of denatured egg albumin have been estimated in a variety of ways in solutions of alkyl sulfate, urea, and guanidine hydrochloride which serve to denature the protein and to keep the denatured protein in solution. In a neutral solution of guanidine hydrochloride it requires 1 cc. of 0.001 M porphyrindin to abolish the SH groups of 10 mg. of coagulated egg albumin as shown by the abolition of the nitroprusside test (Greenstein, 1938). In a neutral solution of Duponol PC, a mixture of long chain alkyl sulfates, 1 cc. of 0.001 M ferrocyanide is formed when 1 cc. of 0.001 M ferricyanide is added to 10 mg. of denatured egg albumin (Anson, 1939a, b). The evidence that ferricyanide is oxidizing only SH groups and all the SH groups is that cysteine is the only amino acid known to reduce ferricyanide under the conditions used, that heat denatured egg albumin treated with iodoacetamide in the absence of denaturing agents no longer reduces ferricyanide in Duponol PC solution, that the amount of ferricyanide reduced by albumin denatured by Duponol PC is within wide limits independent of the concentration of ferricyanide, and the pH, time, and temperature of the reaction (Anson, 1939a, b), that the amount of ferricyanide reduced is the same whether the reaction is carried out in solution of Duponol PC, urea, or guanidine hydrochloride (Anson, 1940, 1941), and that the number of SH groups detected is the same whether one measures the amount of ferricyanide reduced or the amount of ferricyanide, tetraethionate, or p-chloromercuribenzoate needed to abolish the SH groups, as shown by the abolition of the nitroprussi-
side test (Anson, 1940, 1941) or the number of SH groups of neutral native egg albumin titrated by iodine in 1 M KI at 0°C (Anson, unpublished experiments).

Before reagents such as guanidine hydrochloride and alkyl sulfate were introduced, attempts were made to estimate the SH groups of heat coagulated egg albumin which led to SH values that are now known to be about half the correct value. Hopkins (1925) first showed that oxidized glutathione can oxidize some, at least, of the SH groups of denatured egg albumin. When cystine (Minsky and Anson, 1935) and porphyrindin (Kuhn and Desnuelle, 1938) were added to 10 mg. of coagulated egg albumin, the amount of oxidizing agent reduced was equivalent to only 1 cc. of 0.0005 M cysteine.

The first studies of the reactions between ferricyanide and coagulated SH proteins showed that ferricyanide can oxidize tyrosine and tryptophane groups as well as SH groups and that the amount of ferricyanide reduced is greater the higher the concentration of ferricyanide (Minsky and Anson, 1936). Later experiments showed that if the concentration of ferricyanide is not too high, ferricyanide reacts only with the SH groups of denatured egg albumin and not with tyrosine, tryptophane, or disulfide groups (Anson, 1939b), and that the amount of ferricyanide reduced by SH groups alone depends on the concentration of ferricyanide and on the physical state of the protein, the more the protein is aggregated, the less ferricyanide being reduced (Anson, 1939a). Reference was made to earlier viscosity measurements which showed

2 There is no significant change in the sedimentation rate of the albumin as a result of the iodine reaction. Thus the oxidation of the SH groups of native egg albumin by an equivalent amount of iodine does not involve polymerization of the protein. The SH groups of egg albumin can be abolished by reaction of the neutral native protein not only with iodine but also with dilute permanganate or with hydrogen peroxide in thousands of times greater concentration than is needed for the oxidation of the SH groups of denatured egg albumin.

3 When a substance reacts with a protein which is precipitated instead of in solution, the substance has farther to diffuse before it meets a protein particle, it has to penetrate the precipitate, and it reacts with groups whose properties must be changed to some extent by the bonding between molecules in the precipitate. Since a significant fraction of all cellular proteins is not in solution, it would be desirable that the reactions of proteins in the solid state be systematically studied. Some years ago, as a result of some failures in trying to dry crystalline carboxypeptidase without inactivation, I began a study of the denaturation of protein in the solid state. When protein crystals were made insoluble by denaturation procedures the superficial crystalline form was retained (Anson, 1938). The temperature coefficient of denaturation of protein in the solid state was roughly the same as that of denaturation in solution (unpublished experiments). If a solid particle had denatured as a whole, the way a single protein molecule in solution denatures as a whole, the temperature of denaturation would have been almost as sharp as a melting point. When the SH groups of proteins denatured in the solid state were studied by the techniques then available, difficulties were encountered which led to a further study of SH groups. The subject of the SH groups of protein denatured in the solid state has not since been reinvestigated.
that denatured egg albumin can be aggregated even in apparently clear solution and that this aggregation is sensitive to salt (Anson and Mirsky, 1932). Because of the aggregation effect the practical estimation of the SH groups was carried out in the presence of Duponol PC, which acts as a solvent as well as a denaturant. Furthermore, the experiments on the reaction of ferricyanide with the SH groups of denatured egg albumin in the absence of denaturing agents were carried out not with a precipitate but with a solution of denatured egg albumin which reacted much more readily with ferricyanide than the suspensions of coagulated egg albumin previously used. Denaturation was brought about in acid solution and then the denatured protein was cooled and brought into neutral solution. The SH groups of 10 mg. of such a preparation of denatured egg albumin in solution were oxidized 64 per cent by 1 cc. of 0.001 M ferricyanide and oxidized 94 per cent if the ferricyanide concentration was increased 25 times (Anson, 1939b). For the present experiments on heat denatured egg albumin in the absence of denaturing agents, denatured egg albumin was prepared in essentially the same way as previously but even more care was taken to avoid precipitation.

The effect of urea on the SH groups of denatured egg albumin can be shown with the uric acid reagent in neutral solution or with ferricyanide in acid solution. The uric acid reagent oxidizes the SH groups of denatured egg albumin in neutral urea solution (Table II, Experiment 1) but not in alkyl sulfate solution (Experiment 2) or in the absence of denaturing agents (Experiment 3). Guanidine hydrochloride gives a precipitate with the uric acid reagent.

Ferricyanide, a stronger oxidizing agent than the uric acid reagent, oxidizes the SH groups of neutral denatured egg albumin in alkyl sulfate solution (Table II, Experiment 4) or in the absence of denaturing agents (Experiments 5 and 6). In alkyl sulfate solution at pH 4.9, however, ferricyanide oxidizes the SH groups of denatured egg albumin only 21 per cent (Experiment 7). The per cent oxidation is increased from 21 to 86 if urea is present instead of alkyl sulfate at pH 4.9 (Experiment 8). In the absence of denaturing agents denatured egg albumin is insoluble at pH 4.9. At pH 3.9 oxidation by ferricyanide is more complete in guanidine hydrochloride than in urea solution (Experiments 9 and 10).

The blue color formed when the SH groups of a denatured protein are oxidized by the uric acid reagent in neutral urea solution is a convenient measure of the SH content of the protein. The number of SH groups of completely denatured egg albumin, edestin, and tobacco mosaic virus oxidized

4 The SH groups of denatured tobacco mosaic virus in alkyl sulfate solution are not oxidized even by ferricyanide. Recently Dr. Seymour Cohen has found by measurements of sedimentation rates that tobacco mosaic virus in sodium dodecyl sulfate solution is dissociated into molecules about the size of egg albumin. The failure of ferricyanide to oxidize the virus SH groups in sodium dodecyl solution is therefore not due to aggregation.
in neutral urea solution by the uric acid reagent is the same as the number of SH groups detected by ferricyanide titration in guanidine hydrochloride solution by the procedure described elsewhere (Anson, 1941; Anson and Stanley, 1941). Egg albumin and edestin are rapidly denatured in neutral urea solution in the presence of the uric acid reagent. Tobacco mosaic virus, however, is not rapidly denatured by neutral urea and the native virus does not reduce the uric acid reagent in urea solution. The virus, therefore, is denatured in acid urea solution before the estimation of the SH by the uric acid reagent in neutral solution.

Greenstein (1938, 1939) titrating with porphyrindin, a much stronger oxidizing agent than the uric acid reagent, found that fewer SH groups of egg albumin, edestin,

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Denaturant</th>
<th>pH</th>
<th>Oxidant</th>
<th>Amount of denaturant</th>
<th>Oxidation in 10 minutes</th>
<th>Composition of solution</th>
<th>Final volume</th>
<th>Amount of oxidant</th>
</tr>
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<tr>
<td>1</td>
<td>Urea</td>
<td>6.6</td>
<td>Uric acid reagent</td>
<td>100</td>
<td>5.9 gm.</td>
<td>1 Na2HPO4</td>
<td>1</td>
<td>10</td>
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<td>2</td>
<td>Sodium dodecyl sulfate</td>
<td>6.7</td>
<td>“ “</td>
<td>0</td>
<td>10 mg.</td>
<td>3 Na2HPO4</td>
<td>1 Na2HPO4</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Heat</td>
<td>6.7</td>
<td>“ “</td>
<td>Trace</td>
<td>0</td>
<td>1 Na2HPO4</td>
<td>1 Na2HPO4</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>NaDS</td>
<td>6.7</td>
<td>Ferricyanide</td>
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<td>10 mg.</td>
<td>1 Na2HPO4</td>
<td>1 Na2HPO4</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
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<td>6.7</td>
<td>“ “</td>
<td>94</td>
<td>0</td>
<td>1 Na2HPO4</td>
<td>1 Na2HPO4</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>Heat</td>
<td>6.7</td>
<td>“ “</td>
<td>100</td>
<td>0</td>
<td>1 Na2HPO4</td>
<td>1 Na2HPO4</td>
<td>0.1</td>
</tr>
<tr>
<td>7</td>
<td>NaDS</td>
<td>4.9</td>
<td>“ “</td>
<td>21</td>
<td>10 mg.</td>
<td>1 Na2HPO4</td>
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</tr>
<tr>
<td>8</td>
<td>Urea</td>
<td>4.9</td>
<td>“ “</td>
<td>86</td>
<td>1.5 gm.</td>
<td>2.5 NaAc</td>
<td>2 NaAc</td>
<td>0.5</td>
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<td>9</td>
<td>Urea</td>
<td>3.9</td>
<td>“ “</td>
<td>52</td>
<td>1.5 gm.</td>
<td>1 NaAc</td>
<td>(2 × NaHAc)</td>
<td>0.5</td>
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<tr>
<td>10</td>
<td>Guanidine HCl</td>
<td>3.9</td>
<td>“ “</td>
<td>82</td>
<td>1.5 gm.</td>
<td>4 NaHAc</td>
<td>1 NaAc</td>
<td>0.5</td>
</tr>
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</table>
and globin were made titratable by urea than by guanidine hydrochloride. In the light of the present experiments it is unlikely that, as Greenstein supposed, urea failed to "liberate" all the SH groups of his proteins. Denatured SH proteins give a much weaker pink color with nitroprusside in urea than in guanidine hydrochloride solution (Anson, 1941). It is therefore possible that a negative nitroprusside test is not a suitable end-point for the porphyrindin titration in urea solution.

Urea makes the pH of buffers more alkaline. This effect is especially marked with acetate buffers. When a comparison is made between an oxidation in the presence and absence of urea, therefore, a more acid buffer is used in the presence than in the absence of urea so that the pH, as measured by the glass electrode, is the same in both cases (Table II, Experiments 7 and 8).

When urea or guanidine hydrochloride is used a considerable part of the total volume of solution is occupied by the denaturing agent. Thus for a given total volume of solution the concentration of reactants per gram of water is greater in the presence than in the absence of urea and guanidine hydrochloride. An increase in concentration of reactants per gram of water, however, favors oxidation, as has been pointed out. Thus when a comparison is made of a particular oxidation in the presence and absence of urea and guanidine hydrochloride, the reaction is carried out at constant concentrations of reactants per gram of water not per cubic centimeter of solution. The exact amount of water in a saturated urea solution which is available for a protein reaction is not known.

The uric acid reagent not only does not oxidize the SH of denatured egg albumin in neutral alkyl sulfate solution itself but interferes with the oxidation by ferricyanide in alkyl sulfate solution. Tungstate does not interfere with the ferricyanide oxidation. Both the uric acid reagent and tungstic acid, however, slow up the reaction between ferrocyanide and ferric sulfate to form Prussian blue. No study was made of the mechanisms of these inhibitions.

Alkyl sulfates inhibit the oxidation of the SH groups of denatured egg albumin in urea solution by the uric acid reagent. This inhibition is greater the higher the concentration of alkyl sulfate, and it is greater with Duponol PC, a mixture of long chain alkyl sulfates, than with pure sodium dodecyl sulfate. Thus detergents may act as inhibitors as well as solvents.

Hydrolysis.—The effect of hydrolysis in activating the SH groups of denatured egg albumin can be demonstrated with either the uric acid reagent or with ferricyanide. Denatured egg albumin is not oxidized by the uric acid reagent in neutral solution in the presence or in the absence of alkyl sulfate (Table II, Experiments 2 and 3). In contrast, all the SH groups of a peptic hydrolysate of egg albumin are oxidized by the uric acid reagent in neutral solution either in the presence or in the absence of alkyl sulfate (Table III).
At pH 4.8 in a solution of sodium dodecyl sulfate, 1 cc. even of 0.05 M ferricyanide oxidizes 10 mg. of egg albumin only 15 per cent. Under the same conditions the hydrolysate is oxidized 83 per cent (Table III).

78 per cent of the SH in a peptic digest of egg albumin is precipitated by the uric acid reagent, which is a phospho-18-tungstic acid (Wu, 1920), or by saturated ammonium sulfate. The SH which is precipitated is either a part of peptides precipitable by phospho-18-tungstic acid (a more satisfactory precipitant for peptides than tungstic acid) or it is adsorbed to the precipitate which is formed. Free cysteine and cysteine added to a peptic digest of egg albumin are not precipitated by phospho-18-tungstic acid. The 22 per cent of the SH of a peptic digest of egg albumin which is not precipitated by phospho-18-tungstic acid is either free cysteine or SH peptides which are not precipitated by phospho-18-tungstic acid.

Pure SH peptides such as would be desirable for studies of the relation of peptide structure to the properties of the peptides' SH groups have as yet not been isolated from enzymatic digests of proteins. The ease with which SH or its oxidation product, S—S, is estimated should facilitate the isolation of such peptides.

If hydrolyzed egg albumin is allowed to stand in neutral solution, the SH groups are oxidized to S—S by the oxygen of the air. As will be described in a later paper, the total S—S present can then be readily estimated by sulfite plus the uric acid reagent.

**EXPERIMENTAL PROCEDURES**

*Reagents.*—Cysteine hydrochloride—Hoffmann-La Roche. Dissolved in 0.1 N HCl, stored at 0°C., and used promptly.

Urea—Merck's reagent. The SH groups of denatured egg albumin were stable for one-half hour in the presence of the particular sample of urea used. Other samples contained more catalytic impurities.
Guanidine hydrochloride—prepared from purified guanidine carbonate according to Anson (1941).
Duponol PC—du Pont. Stored as a 10 per cent solution.
Sodium dodecyl sulfate—prepared especially by du Pont through the kind offices of Dr. Samuel Lenher. Not available commercially.
Ferricyanide—purified according to Anson (1941).
Uric acid reagent—prepared according to Folin (1934).
Ferric sulfate with gum ghatti—prepared according to Folin and Malmros (1929).

**Egg Albumin.**—Recrystallized by ammonium sulfate or sodium sulfate to constant SH content, dialyzed, and stored frozen. Once crystallized egg albumin always has a low SH content and sometimes the SH content is not raised to the usual value of 1.2 per cent cysteine even by repeated recrystallizations. When two procedures for the estimation of SH are being compared, the same sample of egg albumin should be used for both procedures.

Egg albumin denatured by heating in acid is prepared as follows. To 6 cc. of 2 per cent egg albumin add 1 cc. of 1 N HCl, place in 50°C. bath for 5 minutes, cool in ice water, add slight excess of 1 N NaOH so that the solution is blue to thymolphthalein (about 0.1 cc. 0.1 N NaOH excess per 10 mg. albumin), add water to 12 cc., cool and store in ice water, and use promptly. Egg albumin denatured by trichloracetic acid and washed by decantation before solution with NaOH gives the same results as egg albumin denatured at 50°C.

To prepare digested egg albumin a pH 2.0 solution containing 10 mg. of egg albumin and 1 mg. of crystalline pepsin per cc. of 0.045 N HCl is kept at 65°C. for 1 hour. The vessel is evacuated and filled with 99.8 per cent nitrogen several times before digestion is begun. If digestion is carried out in air, there is a small loss in SH. The hydrolysate gives no precipitate with hot 0.2 N trichloracetic acid. Pepsin itself self-digested at pH 2.0 and 65°C. does not give a nitroprusside test in guanidine hydrochloride solution.

The quantities of reagents used, the volume of solution in which the reaction is carried out, the time of reaction, and the percentage oxidation are given in the tables. The pH was measured by the glass electrode.

The reactions with the uric acid reagent are all carried out at 25°C. After the designated time of reaction the solution is diluted to 10 cc., if it already is not 10 cc., and the blue color formed by the reduction of the uric acid reagent is read either against a standard blue solution formed by the oxidation of 1 cc. of 0.001 M cysteine or against a blue glass calibrated with a standard blue solution. When the blue solution contains urea it is necessary in order to have an optically homogeneous solution either to use a dry colorimeter cup or, if the cup contains residual water, to rinse the cup with a urea solution containing 1 gm. of urea per cc. water.

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*When egg albumin was hydrolyzed completely by hot, concentrated sulfuric acid in air, only 1 cc. of 0.005 M cysteine was found in the hydrolysate of 10 mg. of egg albumin (Mirskey and Anson, 1935). Half the SH was presumably lost by oxidation and by adsorption to humin. During the partial hydrolysis by pepsin under the conditions described there is no oxidation of SH or formation of humin.*
When SH is oxidized by the uric acid reagent, the solutions are prepared and mixed as follows. Into one test tube there are pipetted the uric acid reagent, the 1 cc. of 1 M buffer (except with heat denatured albumin, when 1 cc. of 0.1 M buffer is used), a volume of 0.5 N NaOH equal to the volume of uric acid reagent (this NaOH being added to neutralize the acid reagent), copper sulfate or alkyl sulfate, when they are used, and water to make up a volume 1 cc. less than the final volume desired. 1 cc. of SH solution is pipetted into another test tube. The uric acid reagent solution is poured into the SH solution and the mixed solution poured back and forth. This manner of mixing minimizes formation of turbid solutions and the introduction of catalytic impurities.

When urea is used, it is first placed into a dry test tube to which the uric acid reagent, etc., are then added. The test tube is placed in a 37°C. bath to dissolve the urea and then the solution is brought to 25°C. before being mixed with the SH solution. After the mixing, the mixed solution is centrifuged to hasten the removal of air bubbles.

When both Duponol PC and 5.9 gm. urea are present in 10 cc. solution, some of the Duponol PC comes out of solution in time. Before the colorimetric reading is made, therefore, the solution is warmed to dissolve the Duponol.

When cyanide is added to inhibit oxidation by the uric acid reagent, it is added to the SH solution just before the mixing with the uric acid reagent.

The ferricyanide reactions are all carried out at 37°C. and, except Experiments 7-10 of Table II, are carried out as follows. The reagents and water are mixed in a test tube, the SH solution being added next to last, and the ferricyanide last. 1 cc. of 1 M buffer is used, except with heat denatured egg albumin when 1 cc. of 0.1 M buffer is used. In the experiments with heat denatured egg albumin, furthermore, the ferricyanide is added promptly after the protein. If the heat denatured protein is allowed to stand in the neutral buffer solution, its SH groups become less completely oxidized by dilute ferricyanide. Ferricyanide is added last so that the alkaline heat denatured albumin does not come in contact with the ferricyanide before the protein is neutralized. After the ferricyanide reaction has taken place in 10 cc. for the designated time at 37°C., there are added 0.5 cc. of 2 N H₂SO₄, 0.5 cc. of 10 per cent Duponol PC, 0.5 cc. of 0.1 M ferricyanide or 0.5 cc. of water if the solution already contains concentrated ferricyanide, and 0.5 cc. of ferric sulfate. As previously pointed out (Anson, 1939 b), the Duponol is added to prevent turbidity and the extra ferricyanide to promote the formation of Prussian blue. After 20 minutes the Prussian blue formed is read in red light against either a Prussian blue solution developed from 1 cc. of 0.001 M ferrocyanide or against a blue glass calibrated with a Prussian blue standard.

Experiments 7-10 of Table II are carried out as follows. To a mixture of 0.5 cc. SH solution, 0.5 cc. 1 M buffer, and 0.5 cc. ferricyanide there is added either 0.1 cc. of 10 per cent sodium dodecyl sulfate, 1.5 gm. of urea, or 1.5 gm. of guanidine hydrochloride. The ferrocyanide formed in the alkyl sulfate and urea solutions is estimated as Prussian blue as already described, the solution being diluted to 10 cc. before the addition of ferric sulfate, etc. Guanidine hydrochloride, however, interferes with the complete conversion of ferrocyanide into Prussian blue. So in the experiment with guanidine hydrochloride one measures not the ferrocyanide formed but
the number of SH groups surviving. The protein is precipitated and washed with 0.2 M trichloracetic acid and the protein's SH groups estimated with ferricyanide in neutral Duponol PC solution.

The experiment on the effect of copper sulfate on the oxidation of cysteine by ferricyanide is carried out as follows. To 1 cc. of 0.001 M cysteine there are added 0.3 cc. of a 1 M buffer containing equal parts acetic acid and sodium acetate, and 0.5 cc. of 0.002 M ferricyanide. The brown color of ferricyanide does not disappear. 5 drops of 0.002 M copper sulfate are added. The brown color disappears immediately and is replaced by the weak red of copper ferrocyanide.

The experiment which shows that 78 per cent of the SH of a peptic digest of egg albumin is precipitated by 0.05 M phospho-18-tungstic acid in 1 N H₂SO₄ is carried out as follows. To 2 cc. digest there are added 0.8 cc. uric acid reagent, 5 cc. 2 N H₂SO₄, and water to 10 cc. The precipitate is centrifuged off and its SH content estimated with the uric acid reagent in neutral urea solution. The SH content of the precipitate is the same whether 1 cc. of 0.001 M cysteine is added to the digest or not.

**SUMMARY**

1. Cyanide inhibits the oxidation of the SH groups of cysteine and denatured egg albumin by the uric acid reagent.
2. At pH 4.8 cysteine is oxidized by the uric acid reagent and by ferricyanide in the presence but not in the absence of added copper sulfate.
3. In neutral solution, the uric acid reagent oxidizes the SH groups of denatured egg albumin in the presence of urea but not in the presence of alkyl sulfate or in the absence of denaturing agents.
4. Ferricyanide oxidizes the SH groups of neutral denatured egg albumin even in the presence of alkyl sulfate or, if precautions are taken to avoid aggregation, in the absence of denaturing agents.
5. In acid solution, ferricyanide does not oxidize the SH groups of denatured egg albumin completely. The oxidation is more complete, however, in the presence of urea than in the presence of alkyl sulfate, and more complete in the presence of guanidine hydrochloride than in the presence of urea.
6. The uric acid reagent which does not oxidize the SH groups of neutral denatured but unhydrolyzed egg albumin in the absence of denaturing agents does, under the same conditions, oxidize the SH groups of egg albumin partially hydrolyzed by pepsin.
7. At pH 4.8 in alkyl sulfate solution ferricyanide oxidizes the SH groups of digested egg albumin more completely than the SH groups of denatured but undigested egg albumin.

**REFERENCES**


Anson M. L., 1939a, Science, 90, 256.