SULFHYDRYL GROUPS OF EGG ALBUMIN IN DIFFERENT DENATURING AGENTS

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When egg albumin is denatured there occurs a striking change in its SH groups. This change provides a clue to an understanding of the change in configuration of the egg albumin molecule that takes place during denaturation. A protein can be denatured by many different agents. This is a well known and important characteristic of protein denaturation. In the present investigation our purpose is to discover whether different denaturing agents liberate the same or different numbers of SH groups in egg albumin.

The change in SH groups is readily observed. Native egg albumin does not give a color reaction with nitroprusside and does not reduce ferricyanide; denatured egg albumin gives the color with nitroprusside characteristic of SH groups and immediately reduces ferricyanide (4, 17). This change in the SH groups of egg albumin is an example of the general rule that certain groups in a protein become reactive as a result of denaturation. Other groups, in addition to sulfhydryl, become reactive when egg albumin is denatured. Some of these groups also reduce ferricyanide, though they require a more alkaline medium for their activity than do SH groups. These reducing groups are probably the phenolic groups of tyrosine (20). The SH groups of denatured egg albumin react with iodoacetate (17). Other groups of denatured albumin react with iodoacetate (22). Some of these groups, as yet unidentified, do not react with iodoacetate while the protein is native. There are proteins (in striated muscle and the crystalline lens of the eye) in which, unlike egg albumin, some SH groups are reactive even while the proteins are in the native state. Even while native, these proteins give a color reaction with nitroprusside and reduce ferricyanide (19). In these proteins denaturation produces a marked increase in the number of reactive SH groups. In other proteins (the serum proteins of the horse, for example) no SH groups are detectable either before or after denaturation.1 But in both native and denatured serum proteins disulfide (S—S)

1 Greenstein found that horse serum albumin gives a nitroprusside reaction in presence of a high concentration of guanidine hydrochloride (10, 11). I can confirm this observa-
groups can be shown to be present and they can be estimated after being reduced to SH groups (18). It is then found that there is a larger number of reactive S—S groups in the denatured than in the native serum proteins. The method used for estimating S—S groups in the serum proteins was subsequently used to estimate S—S groups in insulin (23) and lactalbumin (12). Of all the instances of reactive groups appearing in proteins after denaturation the occurrence of reactive SH groups in egg albumin is an example that presents several advantages for investigation: SH groups can be estimated with precision, and the complete absence of reactive groups in native egg albumin makes the increase in number on denaturation especially striking.

The present investigation deals with the effects of three different denaturing agents on the SH groups of egg albumin. The three denaturing agents are urea, guanidine hydrochloride, and the synthetic detergent, Duponol P. C. (a mixture of the C_{10}-C_{18} compounds of the series CH_{2}(CH_{2})_{n} CH_{3}OSO_{3} Na) (1, 3, 8, 21). In each instance the denatured protein remains in solution while the denaturing agent is present.

**Method**

Protein SH groups are estimated by means of their reaction with ferricyanide, as a result of which they are oxidized to S—S groups and ferrocyanide is formed.

\[
2 \text{Protein SH} + 2 \text{ferricyanide} = \text{Protein S—S} + 2 \text{ferrocyanide}
\]

An excess of ferricyanide is added and the quantity of ferrocyanide formed is estimated. This is done by adding ferric sulfate which reacts with ferrocyanide to form Prussian blue which is estimated with a photoelectric colorimeter of the Evelyn type. The intensity of the blue color is a measure of the number of active, protein SH groups. Before adding ferric sulfate it is necessary either to remove the protein or to add some reagent that will keep protein in solution even in presence of ferric sulfate. Both procedures are followed.

For relatively simple SH compounds, such as cysteine and glutathione, the reaction with ferricyanide proceeds stoichiometrically. There is no difficulty in titrating the SH groups of glutathione with ferricyanide (16). The titration is simple and accurate, with a sharp end-point. Ferricyanide reaction; a definite but not intense reaction is obtained. Estimation of the number of SH groups shows that less than 0.1 per cent is present, hardly a significant quantity.
A. E. MIRSKY

has already been used to estimate the SH groups of a protein-denatured globin (20). It was at that time observed that "Whereas the oxidation of SH to S—S by ferricyanide is a definite reaction under suitable conditions (2 SH + 2 ferricyanide = 1 S—S + 2 ferrocyanide) the reaction of the other (reducing) groups (of a protein) with ferricyanide is not so definite. The greater the ferricyanide concentration and the longer the time of reaction, the more oxidation by ferricyanide takes place." At that time ferricyanide was not used to estimate the SH groups of denatured egg albumin. A more cumbersome procedure was followed. Conditions under which the reaction between ferricyanide and the SH groups of denatured egg albumin is precise and definite have now been found; the protein should be dissolved in approximately neutral solutions of urea, guanidine hydrochloride, or Duponol. Under these conditions the reaction goes with great speed. It is completed in less than 1 minute; no more ferricyanide is reduced in 60 minutes than in 1 minute. Nor within wide limits do the concentration of ferricyanide or temperature affect the quantity of ferricyanide reduced. These observations suggest that in the reaction between ferricyanide and egg albumin in neutral solutions of urea, guanidine hydrochloride, or Duponol only the SH groups of the albumin reduce ferricyanide.

There are, as mentioned above, other reducing groups in denatured egg albumin, but those that have been investigated reduce ferricyanide in a slightly alkaline medium only. Furthermore, the reaction of these non-SH groups with ferricyanide is sluggish, there being no definite end-point, and the quantity of ferricyanide reduced depends upon the concentration of ferricyanide present. These groups, then, do not take part in the clearly defined reaction between egg albumin and ferricyanide in a neutral medium.

That the groups of denatured egg albumin which reduce ferricyanide in neutral medium appear to be SH groups and nothing but SH groups can be shown by using the nitroprusside test, especially in conjunction with certain reagents that combine with SH groups. This test in a protein may be considered to be specific for SH groups, for no other groups in a protein are known to give a color reaction with nitroprusside. It is possible that there

2 After most of the experiments on egg albumin in solutions of urea and guanidine described in this paper were completed, Anson discovered the effect of Duponol (1). It was then that Duponol was used in the experiments reported in this paper. Anson found that the amount of ferricyanide reduced by denatured egg albumin in Duponol P C solution is within wide limits independent of the concentration of ferricyanide and the time, temperature, and pH of the reaction. The similar observations on egg albumin in solutions of urea and guanidine hydrochloride described in this paper had already been independently made.
are SH groups which do not give a nitroprusside test and which might react with some reagent other than ferricyanide. A detailed comparison under many different conditions of the color reaction of egg albumin with nitroprusside and the reducing reaction with ferricyanide shows a close correlation between these two reactions. When egg albumin reduces ferricyanide in neutral medium it gives a color with nitroprusside, and when it does not reduce ferricyanide it fails to give a color with nitroprusside. A number of examples of this correlation may be cited:

1. Native egg albumin does not give a color test with nitroprusside; nor does it reduce ferricyanide.

2. When egg albumin is denatured by urea, guanidine hydrochloride, Duponol, or any other agent, it gives a nitroprusside test and also reduces ferricyanide. After the reaction with ferricyanide is completed the albumin no longer gives a nitroprusside test.

3. Heat coagulated egg albumin is treated with ferricyanide and the excess ferricyanide is washed away when the reaction appears to be ended. The albumin no longer gives a test with nitroprusside. Guanidine hydrochloride is then added to the albumin. The albumin now gives a color reaction with nitroprusside and also reduces ferricyanide.

4. Egg albumin denatured by urea is oxidized with ferricyanide and the excess ferricyanide is removed. The albumin no longer gives a test with nitroprusside. The albumin is now treated with guanidine hydrochloride. Neither a nitroprusside test nor reducing action with ferricyanide is observed.

5. When guanidine hydrochloride is added to egg albumin solutions in the pH range from 5.8 to 7.8 precisely the same quantities of ferricyanide are reduced and in no case is a nitroprusside reaction observed when the excess ferricyanide is removed. When guanidine hydrochloride is added to albumin at pH 4.4, 21 per cent less ferricyanide is reduced. Now when the excess ferricyanide is removed and the albumin is tested with nitroprusside in presence of guanidine hydrochloride a slight color reaction is observed. This albumin, brought to pH 7.0, reduces ferricyanide in presence of guanidine hydrochloride. It reduces 16 per cent of the quantity it would have reduced if it had not previously reacted with ferricyanide at pH 4.4.

6. The SH groups of denatured egg albumin react with iodoacetate and iodoacetamide. Egg albumin, so treated, no longer gives a nitroprusside test; nor does it reduce ferricyanide.

7. SH groups of denatured egg albumin, like other SH groups, react with mercuric chloride. After the reaction the albumin neither gives a nitroprusside test nor reduces ferricyanide.
The close correlation between the nitroprusside test and the tendency to reduce ferricyanide makes it unlikely that there are any other groups in egg albumin in addition to SH which reduce ferricyanide in a neutral medium. It is also unlikely, quite apart from the nitroprusside test, that there are any groups other than sulfhydryl in egg albumin which combine with iodoacetate and mercuric chloride and which also reduce ferricyanide in neutral medium. And yet it should be recognized that the existence of such groups has not been completely excluded. Ferricyanide certainly reacts with all SH groups giving a nitroprusside test. But it is possible that there are a few non-SH groups in a protein that react with ferricyanide in neutral medium. In a protein the reactive range of other reducing groups may overlap to a slight extent with the range of activity of SH groups. With this reservation the quantity of ferrocyanide formed can be taken as a measure of the number of reacting SH groups. It remains to be shown that none of the ferrocyanide formed in the reaction between protein and ferricyanide is lost in those cases in which the protein is removed before estimating ferrocyanide. And, in fact, none is lost, for it is found that ferrocyanide added before removing protein is completely recovered when the protein is subsequently removed.

Denaturation

A protein is said to be denatured when it is insoluble in a medium in which it is soluble while still native. Egg albumin denatured by heat is insoluble in water at the isoelectric point, pH 4.7—a medium in which native egg albumin is soluble. The egg albumin in urea, guanidine hydrochloride, or Duponol, which reduces ferricyanide is denatured. It is soluble at the isoelectric point in the presence of urea, guanidine hydrochloride, or Duponol, but when these denaturing agents are removed or diluted with water the protein is found to be insoluble.

Experiments on egg albumin in urea solutions show clearly that liberation of SH groups and formation of insoluble protein are integral parts of the same process. To liberate the maximum number of SH groups 1 gram of urea is added to each 1 cc. of albumin solution. After standing for 60 minutes the albumin reduces no more ferricyanide than it does after standing for only 30 minutes; and within 30 minutes all of the albumin is denatured. This can be shown by diluting the urea solution with water, adjusting the pH to 4.7, and adding one quarter of the volume of saturated (NH₄)₂SO₄ (much less than is needed to precipitate native egg albumin). No protein is left in solution. The correlation between liberation of SH
groups and formation of insoluble protein becomes more apparent when insufficient urea is added to liberate all the SH groups. If the number of SH groups liberated is estimated at different intervals of time after adding urea, it is found that with advancing time more and more groups are liberated so that even after 3 hours in urea and ferricyanide no end-point is reached. Tests for presence of insoluble egg albumin (made by adding water, pH 4.7 acetate buffer, and saturated (NH₄)₂SO₄) show that part of the albumin is insoluble, but that some remains soluble. The soluble and insoluble fractions are separated from each other and both are washed free of ferricyanide. In the insoluble fraction there are no more SH groups; this fraction does not give a reaction with nitroprusside even in presence of guanidine hydrochloride. The soluble fraction still contains SH groups; if this albumin is denatured by adding guanidine hydrochloride an intense nitroprusside reaction is observed. Estimation of the number of SH groups in the albumin of the soluble fraction after denaturation by addition of Duponol shows that per milligram of protein there is the same number of groups as in egg albumin not previously treated with urea and ferricyanide. That fraction of egg albumin in urea and ferricyanide which becomes insoluble (when tested under certain clearly defined conditions) has all of its SH groups liberated, whereas albumin that still is soluble has none of its SH groups liberated. Denaturation of egg albumin by urea is a discontinuous process. A given molecule of protein is either native or denatured. Denaturation of egg albumin by some other agents, it will be shown in other papers, is also an all-or-none process.

When enough guanidine hydrochloride is added to egg albumin to liberate the maximum number of SH groups all of the albumin loses its solubility, when tested in the same manner as in the experiments with urea. In experiments with Duponol a different procedure is followed to demonstrate the altered solubility of albumin. The solution of albumin in Duponol is dialyzed against water for a long time to remove as much Duponol as possible. To the clear dialysate is added 1/10 its volume of saturated ammonium sulfate. This suffices to precipitate all the protein in solution, indicating that Duponol, as well as urea and guanidine, alters the solubility of egg albumin.

**RESULTS**

Since the only amino acid known to have SH groups is cysteine, the SH groups of egg albumin are considered to be part of cysteine, and are accordingly expressed as percentage of cysteine. The results are reproducible to within ±5 per cent.
It can be seen from Table I that the quantities of ferricyanide reduced by egg albumin in urea, guanidine hydrochloride, and Duponol are about the same. Guanidine hydrochloride and Duponol do not liberate any SH groups not liberated by urea, as the following experiment shows: Egg albumin in urea is oxidized by ferricyanide. After the urea and ferricyanide have been washed away, the albumin fails to give a reaction with nitroprusside or to reduce ferricyanide when placed in either guanidine hydrochloride or Duponol.

### Table I

<table>
<thead>
<tr>
<th>Albumin preparation No.</th>
<th>Albumin solution</th>
<th>Albumin</th>
<th>Ferricyanide added</th>
<th>Volume after adding buffer</th>
<th>pH</th>
<th>Denaturing agent added</th>
<th>Temperature</th>
<th>Time of reaction</th>
<th>Quantity ferricyanide formed</th>
<th>SH groups</th>
<th>Cysteine per cent</th>
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<td>I</td>
<td>0.25</td>
<td>18.5</td>
<td>0.005</td>
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<td>6.7</td>
<td>350</td>
<td>37.5</td>
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<td>0.00145</td>
<td>17.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>37.5</td>
<td>30</td>
<td>0.00144</td>
<td>17.3</td>
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<td>0.00144</td>
<td>17.3</td>
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<td></td>
<td></td>
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<td>0.00140</td>
<td>16.8</td>
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<td>(Guanidine hydrochloride)</td>
<td>0.025</td>
<td>18.5</td>
<td>0.005</td>
<td>0.350</td>
<td>6.7</td>
<td>300</td>
<td>25.0</td>
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<td>0.00142</td>
<td>17.0</td>
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<tr>
<td>(Duponol)</td>
<td>0.25</td>
<td>18.5</td>
<td>0.005</td>
<td>1.85</td>
<td>6.7</td>
<td>50</td>
<td>37.5</td>
<td>10</td>
<td>0.00153</td>
<td>18.35</td>
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<td>10</td>
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<td>17.8</td>
<td>0.96</td>
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<tr>
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<td>0.20</td>
<td>20.8</td>
<td>0.005</td>
<td>1.85</td>
<td>6.7</td>
<td>37.5</td>
<td>37.5</td>
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<td>0.00171</td>
<td>20.6</td>
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<td>10</td>
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<td>20.2</td>
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<td>0.00143</td>
<td>17.15</td>
<td>0.98</td>
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</table>

* In this experiment the albumin was precipitated with tungstic acid before estimation of ferrocyanide. In the other experiments with urea, Duponol was added to prevent precipitation of protein during the ferrocyanide estimation.
Three different preparations of crystalline egg albumin were used in the experiments described in this paper. For most of the experiments a single preparation was used. Two other preparations were made to learn whether different samples of egg albumin contain the same number of SH groups when denatured. Of these preparations one (preparation number II of Table I) was made by the method of Kekwick and Cannan (13) and the other (preparation number III of Table I) by La Rosa's method. Preparation III was investigated in the electrophoresis apparatus of Tiselius by Dr. Longsworth. No appreciable quantity of any protein constituent of egg white except egg albumin could be detected. All three preparations of albumin when denatured by Duponol were found to contain the same number of SH groups.

There have been several investigations of the SH groups of egg albumin denatured by urea and guanidine hydrochloride. Rosner estimated SH groups by means of their reaction with iodoacetate (22). He found 0.87 per cent in egg albumin denatured by urea. This is about 10 per cent lower than the result obtained by the reaction with ferricyanide—an entirely different method. Greenstein estimated SH groups by titrating them with porphyrindin, a powerful oxidizing agent (8). For egg albumin in urea he obtained 1.00 per cent SH and for albumin in guanidine hydrochloride 1.28 per cent. The results given by the porphyrindin and ferricyanide methods for the SH groups of albumin in urea are in good agreement. In guanidine hydrochloride the two methods do not agree; in this medium porphyrindin titration gives a much higher value. What seems especially significant in the porphyrindin titrations, and this point has been emphasized by Greenstein, is that different denaturing agents, such as urea and guanidine hydrochloride, liberate different numbers of SH groups. This apparent difference in SH groups seems to be due to a defect in method; porphyrindin may react with reducing groups other than SH in a denatured protein. This possibility was recognized by Kuhn and Desnuelle who first used porphyrindin for titrating protein SH groups (14). They accordingly carried out the reaction at 0° and in an especially careful manner. They placed some confidence in their results on heat coagulated egg albumin because they were in agreement with results obtained by other methods (17, 24). To Greenstein it seemed "hardly probable that the dye (porphyrindin) would react with other types of reducing groups in the protein. Such groups, involving tyrosine and tryptophane radicals, as Mirsky and Anson point out, only begin to make their presence felt at pH 10 and, moreover, react very slowly with ferricyanide and not at all with cystine or phosphotungstate. It is certain in any case that they would not exhibit
a nitroprusside reaction.” The fact that the non-SH reducing groups of
denatured egg albumin do not reduce ferricyanide at pH 7.0 but require
a more alkaline medium does not mean that these groups will fail to reduce
porphyrindin, a more powerful oxidant than ferricyanide, at pH 7.0 (and
in the presence of guanidine hydrochloride). Indeed Kuhn and Desnuelle
point out that porphyrexid (closely related to porphyrindin) oxidizes
thiamin to thiochrome in neutral solution while potassium ferricyanide
requires an alkaline solution for the same oxidation. And although it
may be certain that the non-SH reducing groups of denatured egg albumin
“would not exhibit a nitroprusside reaction” this does not prove that they
do not react with porphyrindin in presence of guanidine hydrochloride.
Greenstein showed that the groups of egg albumin exhibiting a nitroprusside
reaction reduce porphyrindin; but he did not show that the groups not
exhibiting a nitroprusside reaction do not reduce porphyrindin. Denatured
excelsin, he observed, neither gives a nitroprusside reaction nor reduces
porphyrindin (9). On the other hand crystalline papain reduces more
porphyrindin than can be accounted for by its sulfur content (5). There
is then some doubt concerning the estimation of protein SH groups by
titration with porphyrindin. The difference in the quantities of porphy-
rindin reduced by egg albumin in urea and in guanidine hydrochloride
(a difference of 28 per cent) is not due to there being an increased liberation
of SH groups in guanidine hydrochloride, for if this were so, egg albumin in
urea that had been oxidized with ferricyanide would subsequently give a
nitroprusside reaction when dissolved in guanidine hydrochloride—and,
as stated above, a nitroprusside test is not obtained under these conditions.

Anson finds the same number of SH groups (equivalent to a cysteine
content of 1.2 per cent) present in egg albumin denatured by guanidine
hydrochloride and Duponol (2).

To explain why active SH groups appear in egg albumin when it is de-
natured, measurements of SH groups must be combined with other kinds
of information about the protein. Such investigations have already been
carried out and will be described in another paper. The significance of
the measurements made in the present investigation will then become clear,
as will also the conclusion that the same number of active SH groups is
present in egg albumin denatured by urea, guanidine hydrochloride, or
Duponol.

8 "Mit Porphyrexid lässt sich Aneurin in neutraler Lösung zu Thiochrom oxydieren,
was sonst nur noch mit Kaliumferricyanid in alkalischer Lösung gelingt."
4 The validity of SH estimations in proteins by titration with porphyrindin has also
been questioned by Brand and Kassel (6).
SUMMARY

1. The reaction between ferricyanide and egg albumin in solutions of urea, guanidine hydrochloride, and Duponol has been investigated.

2. In neutral medium ferricyanide oxidizes all the SH groups of egg albumin that give a color reaction with nitroprusside. In neutral medium ferricyanide appears to react only with the SH groups of egg albumin. The quantity of ferrocyanide formed can accordingly be considered the equivalent of the number of SH groups in egg albumin detectable with nitroprusside.

3. In solutions of urea, guanidine hydrochloride, and Duponol sufficiently concentrated so that all the egg albumin present is denatured, the same number of SH groups are found—equivalent to a cysteine content of 0.96 per cent.

4. In denaturation of egg albumin loss of solubility (solubility not in presence of the denaturing agent, but solubility examined in water at the isoelectric point) and appearance of reactive SH groups are integral parts of the same process. As denaturation proceeds in urea, SH groups are liberated only in the egg albumin with altered solubility and in this albumin the maximum number of SH groups is liberated. In a molecule of egg albumin either all of its SH groups that give a test with nitroprusside are liberated or none of them are.

EXPERIMENTAL

The egg albumin used in most of these experiments was prepared by La Rosa's method and then recrystallized three times (15). The albumin used in one experiment was prepared by the method of Kekwick and Cannan (13). Before being used a sample of egg albumin was dialyzed in a rocking dialyzer until completely free of ammonium sulfate. Concentration of egg albumin was then determined by drying to constant weight at 105°C. The albumin solution was stored in the cold without preservative for the few days during which it was used. Solutions of ferricyanide were used within 3 or 4 days after being made up and during this time were kept in the dark at 1°C.

Deproteinization

Before estimating the quantity of ferrocyanide formed in the egg albumin solution it is necessary (except in the presence of Duponol) to remove the protein. This is done with tungstic acid. A 10 per cent stock solution of sodium tungstate is acidified whenever tungstic acid is needed. To 1.0 cc. of the sodium tungstate solution are added 40 cc. water, 0.70 cc. of 1 N H₂SO₄, and enough water to bring the volume to 50 cc. In presence of Duponol, ferric sulfate does not precipitate protein.
Prussian blue is formed when ferric sulfate is added to an acidified solution of ferrocyanide. There is a tendency for Prussian blue to precipitate. This can be prevented by adding gum ghatti (7). It is convenient to prepare a solution of ferric sulfate in gum ghatti, as described by Folin and Malmros. To 5 cc. of deproteinized (or Duponol containing) solution are added 0.05 cc. of 0.2 M potassium ferricyanide, 1 cc. of ferric sulfate-gum ghatti and then after 5 minutes, 6.5 cc. water. After standing 5 more minutes Prussian blue is estimated in a photoelectric colorimeter of the Evelyn type, using a red filter (Corning No. 241). A red filter is used because the ferricyanide present does not absorb red light to a significant extent, and ferricyanide is present since an excess is added to the albumin. Still more ferricyanide is added at the time of Prussian blue formation because it was found that when minute quantities of ferrocyanide are being estimated, the amount of Prussian blue formed (in the time interval employed) is increased if ferricyanide is present. With the quantity of ferricyanide added the maximum amount of Prussian blue is formed.

To establish a relationship between intensity of color and quantity of ferrocyanide, Prussian blue is formed in solutions containing known quantities of ferrocyanide. In analytical chemistry standard solutions of ferrocyanide are ordinarily considered to be stable. The solutions required in the present experiments are far more dilute (0.0002 M) than those usually used and these dilute solutions of potassium ferrocyanide are not stable. They must be prepared from more concentrated (0.1 M) stock solutions or from solid potassium ferrocyanide whenever an experiment is done. Prussian blue is formed from known quantities of ferrocyanide under precisely the same conditions as when unknown quantities are present. The 5 cc. of solution, to which 1 cc. of ferric sulfate-gum ghatti subsequently is added, contains between 0.50 and 2.5 cc. of 0.0002 M ferrocyanide. Also included in the 5 cc. of solution are 0.05 cc. 1 N H₂SO₄, 0.05 cc. 0.2 M K ferricyanide, and 2.5 cc. tungstic acid. Of these reagents only ferricyanide influences the color intensity of the blue solution finally obtained. Urea, Duponol, and guanidine hydrochloride have also been added to solutions containing known quantities of ferrocyanide. The quantity of Duponol present in the experiments with egg albumin does not affect the intensity of color when Prussian blue is formed. Urea and guanidine hydrochloride do affect the intensity of color, and it is accordingly necessary to have urea and guanidine hydrochloride in the standard ferrocyanide solutions when these reagents are added to egg albumin.

Reactions between Egg Albumin and Ferricyanide

1. In Urea.—To 0.25 cc. of a 7 per cent albumin solution are added 0.05 cc. 1 M KH₂PO₄-K₂HPO₄ pH 6.7 buffer, 0.05 cc. 0.1 M K ferricyanide, and 350 mg. of urea (100 mg. urea added to each 0.10 cc. of albumin containing solution). A number of these solutions are prepared and kept for various periods of time, some at 25°C., others at 37.5°C. After the reaction with ferricyanide, to each solution are added 10 cc. tungstic acid, 0.4 cc. 1 N H₂SO₄, and enough water to bring the volume to 20 cc. The mixture is filtered and 5 cc. of the clear filtrate are taken for Prussian blue formation. Instead of removing egg albumin by precipitation with tungstic acid, the albumin can be left in
solution if Duponol is added for this prevents protein precipitation when ferric sulfate is added. After the reaction between albumin and ferricyanide, 15 cc. of water and 0.4 cc. 1 N H₂SO₄ are added. As the acid is mixed with the protein solution a fine precipitate appears. This clears up at once when 0.5 cc. of a 10 per cent Duponol solution is added. The solution is brought to a volume of 20 cc. by addition of water. When Duponol is added to a strongly acid mixture of egg albumin and ferricyanide, no reaction between protein SH groups and ferricyanide occurs.

**Effect of Iodoacetamide and Mercuric Chloride.**—To 1 cc. of albumin are added 0.3 cc. phosphate buffer, 0.65 cc. H₂O, 25 mg. iodoacetamide, and 2.0 gm. urea. After standing for an hour at 25°C., 0.5 cc. ferricyanide is added and 30 minutes later the albumin is precipitated with tungstic acid. No Prussian blue forms in the filtrate when ferric sulfate is added. Iodoacetamide does not interfere with Prussian blue formation when it is present in a ferrocyanide solution to which ferric sulfate is added. In another experiment 0.2 cc. of 0.1 M HgCl₂ is added instead of iodoacetamide. No Prussian blue is found in this case either.

**Albumin in Urea Oxidized by Ferricyanide and Subsequently Treated with Guanidine Hydrochloride or Duponol**

To 1.25 cc. albumin are added 0.5 cc. phosphate buffer, 0.25 cc. ferricyanide, and 2 gm. urea. After 30 minutes the albumin is precipitated with tungstic acid and the suspension is centrifuged. The protein precipitate is washed with tungstic acid until it is colorless. To the precipitate are added 0.4 cc. 1 M K₂HPO₄, 1 cc. of a 10 per cent Duponol solution, 0.1 cc. ferricyanide, and water to bring the volume to 15 cc. At 37.5° this mixture forms a clear solution. After 20 minutes the solution is acidified with 1 N H₂SO₄ and diluted with water to 20 cc. Of this solution 5 cc. are taken to test for Prussian blue formation, but no color appears. In another experiment guanidine hydrochloride instead of Duponol is added to the tungstic acid precipitate of albumin. The precipitate is packed down hard in the centrifuge and to it are added 0.3 cc. 1 M K₂HPO₄ and 300 mg. guanidine hydrochloride. A small part of the mixture is tested with nitroprusside. No color is observed. To the rest of the albumin-guanidine hydrochloride mixture is added 0.05 cc. ferricyanide. After 30 minutes at 25°, 10 cc. of tungstic acid, 0.5 cc. H₂SO₄, and water are added to bring the volume to 20 cc. No Prussian blue appears in the filtrate when ferric sulfate is added.

**Test for Completeness of Denaturation.**—To 1 cc. of the albumin solution 1 gm. of urea is added. After the solution has stood at 25° for 30 minutes it is diluted to 10 cc. To 5 cc. are added 0.25 cc. of a 2 M pH 4.7 acetate buffer and 1.25 cc. saturated (NH₄)₂SO₄. The suspension is filtered. Only a slight haze appears in the filtrate when trichloroacetic acid is added.

**Oxidation of Albumin in Insufficient Urea to Produce Complete Denaturation**

To 15.5 cc. of albumin solution are added 1.5 cc. of phosphate buffer, 3.0 cc. of 0.1 M ferricyanide, and 13 gm. of urea. The solution remains at 25° for 20 minutes. A heavy precipitate is formed when 20 cc. of a pH 4.7, 1 M acetate buffer are added and with this
suspension 10 cc. of a saturated ammonium sulfate solution are mixed. A clear supernatant is obtained after centrifuging. The supernatant solution is dialyzed at 1° in a rocking dialyzer against distilled water for 24 hours. It is then completely free of ferricyanide, but somewhat turbid. This fluid is filtered and the protein content of the clear filtrate is determined by drying in an oven at 105°. Each cc. contains 8.52 mg. of protein. Since there are 64 cc. of this solution (561.3 mg. in all) and since the 15.5 cc. of albumin solution used at the beginning of the experiment contained 70.2 mg. per cc. (1088 mg. in all), somewhat less than 50 per cent of the albumin originally present was denatured by urea. SH groups in the dialyzed egg albumin solution are estimated by adding to 2 cc. of the solution 0.05 cc. phosphate buffer, 0.05 cc. of 0.1 M ferricyanide, and 0.5 cc. of a 10 per cent Duponol solution and then proceeding as described below. The albumin precipitated from urea solution by adding acetate buffer is washed with a ½ saturated (NH₄)₂SO₄ solution, removing the washings by centrifuging, until the protein precipitate is free of the yellow color of ferricyanide and the washings contain no protein precipitable with trichloroacetic acid. The protein precipitate is then tested for SH groups with nitroprusside and ammonium hydroxide in the presence of guanidine hydrochloride. The test is negative.

2. In Guanidine Hydrochloride.—300 mg. guanidine hydrochloride are dissolved in 0.25 cc. albumin. The solution remains at 25° for 30 minutes and then to it are added 0.05 cc. of 1 M pH 6.8 potassium phosphate buffer and 0.05 cc. of 0.1 M ferricyanide. After the reaction with ferricyanide the protein is precipitated by adding 10 cc. tungstic acid and 0.4 cc. of 1 N H₂SO₄. The suspension is diluted with water to a volume of 20 cc., shaken, and filtered. Of the filtrate 5 cc. are taken for Prussian blue formation. The conditions of the reaction between ferricyanide and egg albumin in guanidine hydrochloride are varied: the temperature is either 25 or 37.5°; tungstic acid is added to the solution at intervals varying from 5 to 80 minutes after mixing ferricyanide with the albumin; the concentration of ferricyanide added is either 0.1 M or 0.5 M; the quantity of guanidine hydrochloride added is either 200 or 300 mg.; ferricyanide, phosphate buffer, and albumin are mixed together before adding guanidine hydrochloride. None of these variations affects the quantity of ferricyanide reduced. The phosphate buffer used can vary in pH from 6.1 to 7.3, or even 1 M K₂HPO₄ can be used, without affecting the reaction, but if an acetate buffer of pH 4.7 is used, less ferricyanide is reduced.

Effects of Iodoacetamide and Merccuric Chloride.—After dissolving 300 mg. of guanidine hydrochloride in 0.25 cc. of albumin, 0.05 cc. of phosphate buffer is added. In one experiment 20 mg. of iodoacetamide are added to this solution and in another experiment 0.05 cc. of 0.1 M HgCl₂ is added. After standing for an hour at 25°C., 0.05 cc. of ferricyanide is added to each albumin-guanidine hydrochloride mixture and after another hour tungstic acid is added. No blue color appears in the filtrate when ferric sulfate is added.

Recovery of Ferrocyanide Added to Egg Albumin in Guanidine Hydrochloride.—The egg albumin in 0.25 cc. is oxidized by ferricyanide in the presence of guanidine hydrochloride and then precipitated by tungstic acid. The precipitated albumin is separated by centrifuging, washed free of ferricyanide with tungstic acid, and then washed with 0.1 M pH 6.8 phosphate. To the precipitated albumin are added 300 mg. guanidine hydrochloride, 0.05 cc. of 1 M phosphate, 0.05 cc. of ferricyanide, and finally 1 cc. of 0.001 M ferrocyanide. After standing for 10 minutes, tungstic acid and water to bring the volume to 20 cc. are
added and the suspension is filtered. The quantity of Prussian blue formed by the ferrocyanide in 5 cc. of the filtrate is compared with the quantity formed in 5 cc. of a control solution made by adding 1 cc. of ferrocyanide to 10 cc. of tungstic acid, 0.05 cc. of ferricyanide, and 8.5 cc. of water. The galvanometer readings of the colorimeter were practically identical for the two Prussian blue solutions showing that all of the ferrocyanide added to the egg albumin was recovered.

Completeness of Denaturation in guanidine hydrochloride was demonstrated as it was in urea: 300 mg. of guanidine hydrochloride were dissolved in 0.25 cc. of albumin. After standing for 15 minutes, 4 cc. of water, 0.25 cc. of a 2 M pH 4.7-acetate buffer, and 1 cc. of saturated (NH₄)₂SO₄ are added. The precipitate is filtered off. Trichloracetic acid is added to the precipitate. No sign of a protein precipitate is detectable.

3. In Duponol.—To 0.25 cc. of the albumin solution are added 0.05 cc. of pH 6.8 1 M phosphate buffer, 2 cc. of water, 0.5 cc. of a 10 per cent Duponol solution, and 0.05 cc. of ferricyanide. The solution is kept at 37.5°C for 10 minutes. It is then acidified by the addition of 0.4 cc. 1 N H₂SO₄ and diluted to 20 cc. with water. 5 cc. of this solution are taken for Prussian blue formation.

Effects of Iodoacetamide and Mercuric Chloride.—To 1.5 cc. of the albumin solution are added 0.2 cc. of phosphate buffer, 0.5 cc. of Duponol, and either 25 mg. of iodoacetamide or 0.2 cc. of 0.1 M HgCl₂. After 10 minutes at 37°C 0.05 cc. of ferricyanide is added. The solution remains at 37.5°C for 30 minutes before acidifying and diluting to 20 cc. No Prussian blue appears in the filtrate when ferric sulfate is added.

Completeness of Denaturation.—To 2 cc. of albumin solution are added 10 cc. of water and 4 cc. of Duponol. The solution is placed in a cellophane tube and dialyzed against distilled water in a rocking dialyzer for 36 hours at 37°C. At the end of this time the albumin solution remains clear. To 3 cc. of this solution is added 0.05 cc. of a saturated ammonium sulfate solution. A heavy precipitate forms. After filtration, 0.5 cc. of 50 per cent trichloracetic acid is added to the clear filtrate. No turbidity appears.

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