THE SULFHYDRYL GROUPS OF EGG ALBUMIN*

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INTRODUCTION

The first part of this paper describes a number of ways of estimating the SH groups of denatured egg albumin by measuring how much of a particular SH reagent is needed to abolish the SH groups and by measuring how much ferricyanide is reduced by the SH groups. The same titration value is obtained whether the SH groups are abolished by the oxidizing agents, ferricyanide and tetrathionate, or the heavy metal compound, *p*-chloromercuribenzoate; whether the titration is carried out in a guanidine hydrochloride solution or in a solution of Duponol PC, a detergent consisting of long chain alkyl sulfates; whether the abolition of the nitroprusside test or the reduction of ferricyanide is used as proof of the abolition of the SH groups. The same amount of ferricyanide is reduced by denatured egg albumin whether the reduction is carried out in a solution of guanidine hydrochloride, urea, or of Duponol PC. This agreement between the SH values obtained by very different procedures is strong evidence of the validity of the results.

Ferricyanide is a particularly convenient titrating agent. It is readily available and stable. Under the conditions used ferricyanide reacts almost immediately with the SH groups of denatured egg albumin and yet does not react with other protein groups. Altogether, denaturation by guanidine hydrochloride or Duponol PC and oxidation of the SH groups by ferricyanide can be carried out in a few minutes.

It is important to use reagents of suitable purity for the titrations in guanidine hydrochloride solution. I have found that some samples of guanidine hydrochloride and of protein contain impurities which bring about the abolition of SH groups and thus interfere with the nitroprusside test and the SH titrations in guanidine hydrochloride solution. A method has accordingly been worked out for obtaining pure guanidine hydro-

* A brief account of the SH titration methods has already been published (Anson, 1940 *b*).

chloride, and the techniques of the nitroprusside test and the SH titrations in guanidine hydrochloride solution have been so modified as to minimize interference by impurities.

Whether or not the SH groups of native egg albumin react with a particular SH reagent depends on which SH reagent is used. All the SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine and some can be abolished by reaction of the native form of the protein with iodoacetamide (Anson, 1940 *a*) despite the fact that the SH groups of native egg albumin do not give a pink color with nitroprusside (Heffter, 1907; Arnold, 1911), and are not oxidized by cystine (Mirsky and Anson, 1935), ferricyanide (Mirsky and Anson, 1936), or porphyrindin (Kuhn and Desnuelle, 1938). The present experiments show further that *p*-chloromercuribenzoate, which combines firmly with the SH groups of denatured egg albumin, combines with native egg albumin either not at all or at least much more loosely than it combines with denatured egg albumin.

The reactions of iodine and native egg albumin (Anson, 1940 *a*) have now been studied in more detail. It has been found that if a small amount of iodine is added in the cold, the SH groups of neutral native egg albumin are all abolished without oxidation of many of the SH groups beyond the S-S stage and without conversion of many tyrosine groups into di-iodotyrosine groups. If enough iodine is added, the SH groups are oxidized beyond the S-S stage, the S-S groups originally present are oxidized, and the tyrosine groups are converted into di-iodotyrosine groups.

The present pictures of protein structure are not complete enough to provide detailed explanations of the various reactions of the SH groups of different native and denatured proteins with different SH reagents. The facts about the properties of protein groups such as SH groups, however, are important for the development of an adequate theory of protein structure.

The SH titrations in guanidine hydrochloride solution which were worked out with egg albumin can be applied to tobacco mosaic virus. Furthermore, the SH groups of the virus, like the SH groups of egg albumin, can be abolished by reaction of the native form of the protein with iodine. No reaction other than the iodine reaction is known by which the SH groups of native egg albumin and tobacco mosaic virus can be abolished. The discovery of the iodine reaction has thus made possible the study of the biological properties of tobacco mosaic virus which has been modified by oxidation of its SH groups by iodine. The chemical and biological experiments with tobacco mosaic virus, which were suggested by the experiments with egg albumin, will be described in other papers.

Previous Estimations of the SH Groups of Denatured Egg Albumin.—The various procedures which have been used to estimate protein SH groups (Mirsky and Anson, 1935; Kuhn and Desnuelle, 1938; Greenstein, 1938; Anson, 1939) are all similar in principle to the methods used to estimate the SH groups of simple SH compounds such as cysteine and glutathione. SH reagents in general react less readily with protein SH groups than with the SH groups of cysteine. Some SH reagents, furthermore, can, under suitable conditions, react with protein groups other than SH groups. The problem, therefore, in the estimation of the SH groups of unhydrolyzed protein is to find conditions under which the SH reagent reacts with all the protein SH groups and no other groups. These conditions have apparently been fulfilled in the estimation of the SH groups of egg albumin by two methods which were developed from the earlier work. In the first method, one measures how much porphyrindin has to be added to denatured egg albumin in guanidine hydrochloride solution so that all the protein SH groups are oxidized and the protein no longer gives a pink color with nitroprusside (Greenstein, 1938). In the second method, one measures how much ferricyanide is reduced by denatured egg albumin in a solution of the detergent, Duponol PC (Anson, 1939).

Ferricyanide and porphyrindin are added to denatured rather than to the native egg albumin because native egg albumin does not reduce ferricyanide and porphyrindin at all. Guanidine hydrochloride or Duponol PC are added because in the absence of such substances not all the SH groups even of denatured egg albumin are rapidly oxidized by dilute ferricyanide and porphyrindin. The nitroprusside test is carried out in guanidine hydrochloride solution but not in Duponol PC solution because the SH groups of denatured egg albumin give a strong pink color with nitroprusside in guanidine hydrochloride solution but only a negligible pink color in Duponol PC solution. The ferricyanide reduction can be carried out in guanidine hydrochloride and urea solution as well as in Duponol PC solution but the estimation of the ferrocyanide formed as Prussian blue is less convenient than when the reaction is carried out in Duponol PC solution.

Various tests were carried out by Greenstein and myself to show that ferricyanide and porphyrindin under the conditions used react specifically and completely with the SH groups of denatured egg albumin. Since no one of these tests is conclusive, the validity of the SH estimations has been tested by comparing the results obtained by different procedures.

SH Titrations in Guanidine Hydrochloride Solutions.—In the porphyrindin titration as originally carried out (Greenstein, 1938) guanidine hydrochloride is added to a neutral protein solution, the solution is allowed to stand

45 minutes, porphyrindin is added to the protein denatured by the guanidine hydrochloride, and finally nitroprusside and ammonia are added to see whether enough porphyrindin has been added to oxidize all the SH groups.

The new titrations in guanidine hydrochloride solution are carried out as follows. To 0.5 cc. of 2 per cent native egg albumin or tobacco mosaic virus there are added 0.1 cc. of neutral phosphate buffer, 0.5 cc. of ferricyanide, tetrathionate or *p*-chloromercuribenzoate[1] solution, and 1.2 gm. of guanidine hydrochloride of tested purity. 3 minutes later test is made for the abolition of the SH groups either by seeing whether the protein gives a nitroprusside test in the presence of dilute cyanide or by seeing whether the protein can still reduce ferricyanide in Duponol PC solution. The concentration of titrating agent is found which just suffices to abolish the SH groups.

The new titrations differ from the porphyrindin titration in that different titrating agents are used; interference by impurities is minimized by using especially purified guanidine hydrochloride, adding the titrating agent before the guanidine hydrochloride, and carrying out the nitroprusside test in the presence of cyanide; the ferricyanide reduction test as well as the nitroprusside test is used to prove the abolition of SH groups; and the whole titration is carried out in the presence of phosphate buffer. I shall now discuss the reasons for the changes which have been made.

In the new titrations ferricyanide, tetrathionate, and *p*-chloromercuribenzoate are used as titrating agents instead of porphyrindin. The substitution of the ferricyanide and tetrathionate for porphyrindin makes the titration in guanidine hydrochloride solution safer and much more convenient. Porphyrindin is hard to prepare, unstable, and a dangerously strong oxidizing agent. Although porphyrindin reacts first with the SH groups of denatured egg albumin in guanidine hydrochloride solution it also in time reacts with other groups. Ferricyanide and tetrathionate are readily available and are weaker oxidizing agents than porphyrindin.

The inclusion of mercuribenzoate as a titrating agent provides a good test for the SH specificity of the titration. Whereas porphyrindin, ferricyanide, and tetrathionate oxidize SH to S-S, mercuribenzoate combines with but does not oxidize SH groups. It is conceivable that the oxidizing agents might oxidize protein groups other than SH groups or that mercuribenzoate might combine with groups other than the SH groups. The SH group, however, is the only protein group known to react with both oxidizing agents and heavy metal compounds.

Formaldehyde abolishes the SH groups of denatured egg albumin in guanidine hydrochloride solution only if the formaldehyde is added in great excess. Formaldehyde cannot, therefore, be used as a titrating agent.

The following observations show that some samples of guanidine hydrochloride contain impurities which bring about the abolition of SH groups and so interfere with the

[1] *p*-chloromercuribenzoate has been used as an SH reagent by Hellerman (1937; 1939).

nitroprusside test and that this interference can be diminished by cyanide. 5 mg. of denatured egg albumin gives a strong pink color with nitroprusside in guanidine hydrochloride solution. I have found, however, that the pink color obtained is much stronger with some samples of commercial guanidine hydrochloride (Eastman or Hoffman-La Roche) than with others. When a guanidine hydrochloride is used which gives a weak color, then the color is weaker the more guanidine hydrochloride is used and the longer the denatured protein is allowed to stand in guanidine hydrochloride solution before the addition of nitroprusside. A sample of guanidine hydrochloride which gives a weak color gives a strong color if it is first recrystallized. If 1 drop of 0.1 N cyanide is added to the protein solution before the addition of guanidine hydrochloride, then a strong nitroprusside test is obtained with all samples of guanidine hydrochloride. Even when a strong nitroprusside test is obtained without cyanide, cyanide slows up the rate of fading of the pink color. On the other hand, the rate of fading can be enormously increased by adding an amount of copper sulfate equivalent to only 10 per cent of the SH groups present. The results which have been summarized do not definitely prove how the impurities in guanidine hydrochloride bring about the abolition of SH groups. They suggest, however, that the impurities are in part, at least, heavy metal compounds which catalyze the oxidation of the SH groups of denatured egg albumin by oxygen and that cyanide inhibits this oxidation of protein SH groups by oxygen by combining with the heavy metal impurities. It is known that heavy metal compounds can catalyze the oxidation of the SH of cysteine (references in Bernheim and Bernheim, 1939) and of denatured egg albumin (Rosenthal and Voegtlin, 1933) by oxygen.

The cyanide added to diminish the effects of impurities in the nitroprusside test is too small in amount to cause any appreciable reduction of S-S to SH. Cystine and denatured egg albumin whose SH groups have been oxidized to S-S groups do not give any color with nitroprusside in guanidine hydrochloride solution even when 1 drop of 0.1 N cyanide is added. The ease with which the S-S groups of a denatured protein are reduced by cyanide varies from protein to protein. 1 drop of 0.1 N cyanide is safe for those proteins which I have tried, but it may not be safe for all proteins.

The following experiments show that impurities in guanidine hydrochloride reduce the amount of the ferricyanide needed to abolish the SH groups of denatured egg albumin, and that the effect of impurities is much less in the new titration procedure in which the titrating agent is added before the protein is denatured by guanidine hydrochloride than in the old titration procedure in which the protein is allowed to stand in guanidine hydrochloride solution before the addition of the titrating agent. When satisfactory guanidine hydrochloride is used the amount of ferricyanide needed to abolish the nitroprusside test is the same whether the ferricyanide is added before the addition of guanidine hydrochloride or 30 minutes after the addition of guanidine hydrochloride. With one poor sample of Eastman guanidine hydrochloride (not the worst), nine-tenths the normal amount of ferricyanide was required to abolish the nitroprusside test if the ferricyanide was added before the addition of guanidine hydrochloride, but only five-tenths the normal amount if the ferricyanide was added 30 minutes later.

The discrepancy between the amount of ferricyanide needed to abolish the SH groups of egg albumin when the ferricyanide is added before the guanidine hydrochloride and when it is added 30 minutes after the guanidine hydrochloride can be used as a test for the purity of the guanidine hydrochloride. When such a test is applied to commercial guanidine hydrochloride, usually, in my experience, the product is found to be unsatis-

factory. Guanidine hydrochloride cannot be purified by recrystallization without great loss. I have therefore found conditions for the effective and economical purification of guanidine carbonate. Guanidine hydrochloride prepared from purified guanidine carbonate is satisfactory for SH titrations.

Greenstein (1938) found that the SH groups of his egg albumin were stable in neutral guanidine hydrochloride solution. His sample of guanidine hydrochloride was therefore satisfactory.

Although one can always make sure that one has pure guanidine hydrochloride and pure egg albumin, not all proteins can readily be obtained in as pure a state as egg albumin. With some proteins it is particularly important to have a titration procedure like the present one which minimizes the effects of impurities and to carry out tests for the presence of impurities. The origin of the present detailed experiments with guanidine hydrochloride, in fact, was my inability to obtain a constant value for the SH content of tobacco mosaic virus when different samples of guanidine hydrochloride and virus were used.

The same titration value is obtained if the nitroprusside test on the albumin treated with the titrating agent is carried out almost immediately after the addition of the titrating agent and the guanidine hydrochloride or 30 minutes after the addition of the titrating agent and guanidine hydrochloride. The waiting has therefore been eliminated and the time needed for the titration very much shortened.

Instead of using the disappearance of the nitroprusside test as an indication that all the SH groups have been abolished one can use the failure to reduce ferricyanide. After the titrating agent and guanidine hydrochloride have been added to the protein, the protein is precipitated and washed with trichloracetic acid, the precipitate is dissolved in neutral Duponol PC solution, ferricyanide is added, and a test is made for ferrocyanide. If either the nitroprusside test or the ferricyanide test for SH were insensitive or not specific for SH then different titration values would be obtained by using these two very different SH tests for the end point. Since the nitroprusside test is more convenient than the ferricyanide reduction test if the titration is carried out in guanidine hydrochloride solution, the ferricyanide reduction test is used not as a routine procedure but only as a check on the validity of the titrations.

The whole SH titration is in all cases carried out in a neutral solution buffered with phosphate. Phosphate establishes a reproducible pH. In the absence of phosphate, furthermore, a pink color is formed immediately on the addition of nitroprusside to a neutral or even slightly acid guanidine hydrochloride solution of denatured protein and the color becomes stronger on the subsequent addition of ammonia. Phosphate conveniently prevents the formation and fading of the pink color before the addition of ammonia.

Cyanide must not be present during the titrations with ferricyanide or mercuribenzoate despite the fact that it is desirable to have cyanide present during the nitroprusside test. For cyanide combines with mercuribenzoate and inhibits the oxidation of protein SH groups by ferricyanide. The mechanism of this inhibition has not been studied. It is possible that the oxidation of protein SH groups by ferricyanide is catalyzed by heavy metal impurities which combine with cyanide. Dilute cyanide does not prevent

the oxidation of the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution by tetrathionate. (Since submitting this paper I have found that cyanide inhibits almost completely the oxidation of SH groups by ferricyanide, tetrathionate, and the uric acid reagent provided the concentration of cyanide is high enough and that copper and zinc ions promote these oxidations.)

In agreement with the results previously obtained by the porphyrindin titration method the equivalent of 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is needed to abolish the SH groups of 10 mg. denatured egg albumin in guanidine hydrochloride solution whether the abolition of the nitroprusside test or the failure to reduce ferricyanide is used as an end point. It should be emphasized that the agreement between the new titrations and the porphyrindin titration in its original form exists only when the samples of protein and guanidine hydrochloride used happen to be free of impurities which interfere with the original titration method much more than they do with the new methods.

Rate and Completeness of SH Reactions in Neutral Guanidine Hydrochloride Solution.—As we have seen, if 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is added to 10 mg. of denatured egg albumin in neutral solution and ammonia and nitroprusside are added 3 minutes later, no pink color is obtained. The question arises, does the SH reagent react with the protein SH groups in the neutral solution, or after the addition of ammonia, or is the colored compound of the nitroprusside test formed but destroyed by the SH reagent before it can be observed? Ferricyanide and mercuribenzoate can rapidly destroy the color formed in the nitroprusside reaction. The experiments designed to answer this question show that the abolition of the SH groups in neutral solution by ferricyanide and mercuribenzoate is completed in 3 minutes but that part of the tetrathionate reaction takes place after the addition of ammonia.

The following experiments show that the oxidation of the SH groups by ferricyanide takes place in the neutral solution. After the addition of the ferricyanide to the neutral protein solution the solution is colorless, indication of reduction of the brown ferricyanide to the colorless ferrocyanide. If the protein which has been treated with ferricyanide is precipitated and washed with trichloracetic acid, a protein precipitate is obtained which is free of ferricyanide and has been exposed to ferricyanide in neutral but not in alkaline solution. The SH groups of this protein have been abolished. The protein gives no nitroprusside test when dissolved in guanidine hydrochloride solution and does not reduce ferricyanide in neutral Duponol PC solution.

Further experiments show that tetrathionate, like ferricyanide, can oxidize all the SH groups of denatured egg albumin in neutral solution but that the oxidation by tetrathionate is slower than the oxidation by ferricyanide and so is not complete in 3 minutes.

The trichloracetic acid precipitate of the albumin treated for 3 minutes with tetrathionate in neutral guanidine hydrochloride solution still gives a moderately strong nitroprusside test in guanidine hydrochloride solution and reduces about half as much ferricyanide in Duponol solution as untreated albumin. Since no nitroprusside test is obtained if ammonia and nitroprusside are added directly after 3 minutes, part of the tetrathionate reaction responsible for the abolition of the nitroprusside test must take place after the addition of ammonia. If the tetrathionate is allowed to stand 30 minutes in the neutral guanidine hydrochloride solution before the addition of trichloracetic acid, then it is found that the SH groups have all been abolished by the reaction in neutral solution.

Finally, the evidence that mercuribenzoate combines with the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution. If first 1 cc. of 0.001 M mercuribenzoate and then 1 cc. of 0.001 M ferricyanide are added to 10 mg. of denatured egg albumin in neutral guanidine solution, the brown color of the ferricyanide persists. If the mercuribenzoate had not combined with and protected the SH groups, the ferricyanide would have been reduced to colorless ferrocyanide. When mercuribenzoate combines with SH groups of urease it similarly protects them from oxidation by porphyrindin (Hellerman, 1939).

The compound between mercuribenzoate and the SH groups of denatured egg albumin in neutral guanidine hydrochloride solution is dissociated by trichloracetic acid. If denatured egg albumin which has combined with mercuribenzoate is precipitated with trichloracetic acid and dissolved again with guanidine hydrochloride it gives about as strong a nitroprusside test as egg albumin which has never been exposed to mercuribenzoate.

Measurement of Ferricyanide Reduction in Guanidine Hydrochloride and Urea Solution.—1 cc. of 0.001 M ferrocyanide is formed when ferricyanide is reduced by 10 mg. of denatured egg albumin in Duponol PC solution (Anson, 1940 *a*) or, as in the present experiments, in guanidine hydrochloride or urea solution. The amount of ferrocyanide formed is within wide limits independent of the ferricyanide concentration.

The estimation of SH groups by ferricyanide reduction is more convenient in Duponol PC than in guanidine hydrochloride or urea solution. Duponol PC, unlike guanidine hydrochloride and urea, prevents the precipitation of denatured egg albumin by the acid ferric sulfate added for the estimation of ferrocyanide as Prussian blue. Duponol PC interferes with the development of Prussian blue less than guanidine hydrochloride and in neutral solution denatures egg albumin more rapidly than urea.

Denaturation in neutral urea solution is slow and egg albumin loses some of its SH groups on standing in neutral urea solution if ordinary commercial urea is used and the urea solution does not contain cyanide. In the present experiments, therefore, denaturation by urea is brought about in acid solution in which denaturation is rapid and SH groups are more stable. When the acid urea solution containing 10 mg. of denatured egg albumin is neutralized, 1 cc. of 0.001 M ferricyanide is added. 1 cc. of 0.001 M ferrocyanide is formed and the protein when precipitated with trichloracetic acid and redissolved with guanidine hydrochloride gives no nitroprusside test. The nitroprusside test of untreated egg albumin in guanidine hydrochloride solution is much more intense than the test in urea solution.

I have not been able to confirm the conclusion of Greenstein (1938) that urea "liberates" fewer SH groups from egg albumin than guanidine hydrochloride. Even if 1 cc. of 0.001 M ferricyanide and urea are added to a neutral solution of 10 mg. of egg albumin which has not been treated with acid, the protein after being precipitated by trichloracetic acid no longer gives a pink color with nitroprusside in guanidine hydrochloride solution.

As will be described elsewhere, the SH groups of egg albumin can be estimated by the blue color given with the uric acid reagent, the SH value being the same as that obtained by the present methods. When unhydrolyzed albumin is used the reaction is carried out in urea solution. When albumin partially hydrolyzed by pepsin or acid is used, the presence of urea is not necessary.

Urea promotes the oxidation not only of the SH groups of denatured egg albumin but also the oxidation of free cysteine, tyrosine, and tryptophane. Partial hydrolysis "activates" not only SH groups but the few other protein groups I have tried.

SH Titrations in Duponol PC Solution.—In neutral Duponol PC solution as in neutral guanidine hydrochloride solution 1 cc. of 0.001 M ferricyanide, tetrathionate, or mercuribenzoate is required to abolish the SH groups of 10 mg. of denatured egg albumin.

After the SH groups of denatured egg albumin have been abolished by ferricyanide or tetrathionate, the protein when precipitated by trichloracetic acid and redissolved in guanidine hydrochloride solution no longer gives a nitroprusside test. As in guanidine hydrochloride solution, the ferricyanide reaction is more rapid than the tetrathionate reaction and cyanide interferes with the ferricyanide reaction but not with the tetrathionate reaction.

After mercuribenzoate has combined with all the SH groups of denatured egg albumin in neutral Duponol PC solution the protein no longer reduces dilute ferricyanide. Thus when the SH estimation is carried out in Duponol PC solution as when it is carried out in guanidine hydrochloride solution, it is possible to titrate the SH groups with both an oxidizing agent and a heavy metal compound and to use both the nitroprusside reaction and the ferricyanide reduction as tests for the abolition of the SH groups.

The ferricyanide reduction test should not be used after the tetrathionate reaction because the decomposition products formed from tetrathionate in acid solution reduce ferricyanide. The nitroprusside test should not be used after the mercuribenzoate reaction because trichloracetic acid dissociates the compound between mercuribenzoate and SH.

The Reactions of Iodine and Native Egg Albumin.—Despite the fact that native egg albumin does not react with nitroprusside, ferricyanide, or porphyrindin, all the SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine. Native egg albumin which has reacted with iodine no longer gives a nitroprusside test when denatured

nor does it reduce ferricyanide in Duponol PC solution (Anson, 1940 *a*). I have now studied the reactions between iodine and neutral native egg albumin in somewhat more detail, mainly in order to compare the egg albumin reactions with the reactions between neutral native tobacco mosaic virus and iodine, which will be described elsewhere.

By adding iodine in acid solution it is possible to oxidize the SH groups of native egg albumin without converting the tyrosine groups into di-iodotyrosine groups. Iodine abolishes the SH groups of native egg albumin even at pH 3.2 (Anson, 1940 *a*). At pH 3.2 iodine does not react with free tyrosine, or with the proteins pepsin (Herriott, 1937), and chymotrypsinogen (Anson, 1940) which contain tyrosine but not cysteine. The present experiments show that it is also possible in neutral solution to oxidize the SH groups of native egg albumin without converting many tyrosine groups to di-iodotyrosine groups or oxidizing many of the SH groups beyond the S-S stage.

If 1.3 cc. of 0.001 N iodine is added to 10 mg. of native egg albumin at 0°C., all the iodine is absorbed as shown by a negative starch test. All the SH groups are abolished as shown by a negative nitroprusside test in guanidine hydrochloride solution. Theoretically it takes 1 cc. of 0.001 N iodine to oxidize the SH of 10 mg. of egg albumin to S-S. The excess 0.3 cc. of 0.001 N iodine actually added is not sufficient to cause much further oxidation of the sulfur groups to RSOH, RSO_2H, or RSO_3H, or to convert many tyrosine groups into di-iodotyrosine groups. The 10 mg. of egg albumin treated with 1.3 cc. of 0.001 N iodine still gives a strong nitroprusside test in guanidine hydrochloride solution, if the protein is exposed to strong cyanide in alkaline guanidine hydrochloride solution before the addition of nitroprusside, indicating S-S groups which are reduced to SH by alkaline cyanide. The 10 mg. of egg albumin which has absorbed 1.3 cc. of 0.001 N iodine still gives a strong purple color when boiled with Millon's reagent. Free di-iodotyrosine (Vaubel, 1900) and, as Harrington and Neuberger (1936) have shown, insulin whose tyrosine groups have been iodinated do not give a color with Millon's reagent.

Since submitting this paper I have found conditions under which the absorption of only 1 cc. of 0.001 N iodine by 10 mg. of native egg albumin brings about the abolition of the nitroprusside test in guanidine hydrochloride solution.

If in the reaction between native egg albumin and iodine, the concentration of iodine and the time and temperature of the reaction are high enough, then all the SH and S-S groups are oxidized beyond the S-S stage, as shown by a negative cyanide-nitroprusside test, and all the tyrosine groups are converted into di-iodotyrosine groups, as shown by a negative Millon test. The conditions for abolishing the cyanide-nitroprusside test are roughly the same as those for abolishing the Millon test.

I have not done any experiments to find out whether the iodine added to

native egg albumin reacts with any groups other than the SH and tyrosine groups.

Egg albumin whose SH groups have been abolished by iodine does not abolish the SH groups of untreated egg albumin in neutral guanidine hydrochloride solution.

Reactions of p-Chloromercuribenzoate with Cysteine and Native Egg Albumin.—In this section it will be shown that mercuribenzoate combines with native egg albumin either not at all or at least much more loosely than it combines with cysteine or with the cysteine in denatured egg albumin.

I have found that the compound between cysteine and mercuribenzoate, like the compound between cysteine and aldehyde (Schubert, 1936) and the cysteine in native egg albumin,[2] does not give a nitroprusside test or reduce ferricyanide but does reduce iodine. Thus the nitroprusside and ferricyanide tests cannot be used to find out whether mercuribenzoate has combined with the SH groups of native egg albumin because these groups do not react with nitroprusside and ferricyanide even when they are not combined with mercuribenzoate. On the other hand, the iodine reaction cannot be used either because SH reduces iodine even when it is combined with mercuribenzoate. I have accordingly used an indirect procedure involving the addition of free cysteine. If mercuribenzoate added to egg albumin is tightly bound to the protein, it cannot combine with added cysteine and the added cysteine is then free to reduce ferricyanide.

If first 1 cc. of 0.001 M cysteine and then 1 cc. of 0.001 M ferricyanide are added to 10 mg. of either native egg albumin or to denatured egg albumin in Duponol PC solution, the ferricyanide is reduced by the cysteine. In the absence of cysteine, native egg albumin does not reduce ferricyanide under any conditions and denatured egg albumin does not reduce ferricyanide under the conditions used, namely, low temperature, dilute ferricyanide, and short time of reaction.

If 1 cc. of 0.001 M mercuribenzoate is added to the *native* albumin before the addition of cysteine and ferricyanide, the ferricyanide is not reduced. This shows that the cysteine has combined with the mercuribenzoate. Either the mercuribenzoate does not combine with the native protein or it is rapidly withdrawn from its combination by the addition of cysteine. In contrast, if the mercuribenzoate is added to *denatured* egg albumin before the addition of cysteine and ferricyanide, the ferricyanide is reduced. Mercuribenzoate remains attached to the SH groups of denatured egg albumin, for a short time at least, even if cysteine is added.

SH Groups and Protein Structure.—The present results and indeed all the work on the SH groups of egg albumin and other proteins show that a

[2] I do not mean to suggest that the SH in native egg albumin is linked to aldehyde or heavy metal or in any other way. It seems to me more likely on the basis of the present inconclusive evidence that the SH groups of native egg albumin are not linked.

reagent which reacts with the SH groups of free cysteine may or may not react with cysteine bound in a protein. Whether or not the reaction takes place depends on what SH reagents and proteins are used, on the concentration of these substances and the time, temperature, and pH of the reaction, on whether the protein is native or denatured, on whether the solution of denatured protein contains substances such as guanidine hydrochloride or Duponol PC, and on whether the solution contains catalysts such as zinc and copper salts or inhibitors such as cyanide.

It would, of course, be desirable to be able to explain the now rather extensive experimental results in terms of some theory of protein structure. The facts, however, although they lead to vague general conclusions about the structural changes involved in denaturation, do not as yet provide proof of any definite, detailed picture of the structural relationships of SH groups in native and denatured proteins. *A priori*, a protein SH group may fail to react with an SH reagent because the protein SH group is inaccessible, or bound, or made unreactive by neighboring protein structure. *A priori*, several of these factors may operate at once, or one factor may be decisive under one set of conditions, another factor decisive under a different set of conditions. As more facts accumulate the arbitrary assumptions which can be made in connection with the three kinds of structural theories become more and more restricted.

It should be remembered that in some proteins S-S (Walker, 1925) and tyrosine (Mirsky and Anson, 1936) groups which are not detectable in the native protein are detectable by the same tests in the denatured form of the protein. The problem of how denaturation and other changes in protein structure produce changes in the properties of protein groups is not peculiar to SH groups.

It should also be remembered that the SH groups of cysteine are more readily oxidized in neutral than in acid solution and that the SH groups of cysteine are more readily oxidized than the SH groups of glutathione (Anson, 1939). Thus molecular structure can greatly influence the properties of SH groups even in relatively simple SH compounds.

EXPERIMENTAL

Reagents.—Egg albumin is thrice recrystallized with ammonium sulfate, dialyzed, and stored frozen.

Duponol PC (Du Pont) is stored at room temperature as a filtered 10 per cent stock solution.

A 5 per cent solution of ground sodium nitroprusside is made fresh daily and stored in ice water. Nitroprusside dissolves slowly unless it is first ground.

The phosphate buffer consists of equal parts of 1.0 M Na_2HPO_4 and NaH_2PO_4.

For the ferricyanide titration reagent grade potassium ferricyanide is used. When the amount of ferrocyanide formed from ferricyanide is measured either a correction is made for the ferrocyanide present in commercial ferricyanide or the ferrocyanide is removed by oxidation with bromine (Anson, 1939). Bromine in addition to oxidizing ferrocyanide to ferricyanide also brings about some other reactions which result in darkening of the solution. This darkening is greater in the original procedure in which the bromine is added step-wise than in the following simpler procedure in which the bromine is added in dilute solution all at once, and in excess. 0.6 M ferricyanide is made up and centrifuged to remove insoluble matter. The ferrocyanide impurity is estimated by adding to 2 cc. ferricyanide solution 7 cc. of water, 0.5 cc. of 2 N sulfuric acid, and 0.5 cc. of ferric sulfate solution. The amount of red light absorbed by the Prussian blue formed is compared with the red light absorbed by a known amount of ferrocyanide in the absence of ferricyanide. Saturated bromine water is diluted 75 times with water and assumed to be 0.005 N which allows for a 10 per cent loss on dilution. If it is desired to know the concentration of bromine in the dilute solution accurately, an excess of iodide is added to a sample and the iodine liberated by bromine is titrated with thiosulfate. Twice the amount of bromine theoretically needed to oxidize the ferrocyanide present is added to the ferricyanide solution and water is added to make the ferricyanide 0.4 M. After the solution has stood 20 minutes the excess bromine is removed by aeration and the solution is allowed to stand overnight before being used. The purified ferricyanide is stored in the cold in a dark bottle. Since in the course of months ferrocyanide forms in the ferricyanide solution the ferricyanide solution is occasionally tested for ferrocyanide with ferric sulfate.

Ferric sulfate containing gum ghatti is prepared according to Folin and Malmros (1929).

A stock 0.1 M thiosulfate solution containing 0.1 gm. sodium carbonate per liter is standardized with the iodine formed by the reaction between iodide and iodate (Peters and Van Slyke, 1932).

The stock 0.1 iodine solution contains 0.18 N Kl. The stock 0.1 N iodine previously used (Anson, 1940*a*) contained only 0.12 N Kl and lost some iodine on dilution with water. The iodine solution is first made up roughly and then titrated with thiosulfate.

Tetrathionate is formed by adding just enough thiosulfate to iodine to abolish the starch test. It is made up just before being used.

p-chloromercuribenzoic acid is prepared according to the directions of Whitmore and Woodward (1932) and dissolved as the sodium salt. The solution is stable for a few days at least if stored frozen. I am indebted to Dr. Leslie Hellerman for the mercuribenzoic acid.

The guanidine hydrochloride used in the present experiments was a satisfactory batch of the Eastman product. Since guanidine hydrochloride is very hygroscopic it is first dried in a desiccator, then distributed in a number of small containers which are tightly stoppered, and stored in the cold or in some dry atmosphere.

Most samples of the commercial guanidine hydrochloride have not proved satisfactory. Good guanidine hydrochloride should dissolve in an equal weight of water to give a clear and colorless solution. The color given with egg albumin and nitroprusside in guanidine hydrochloride solution should be the same as when guanidine hydrochloride recrystallized from water is used and should not be increased if 1 drop of 0.1 N cyanide is present. The rate of fading of the color should not be much greater than that obtained

with recrystallized guanidine hydrochloride. Letting the albumin stand in neutral guanidine hydrochloride solution for 30 minutes before the addition of nitroprusside should not decrease the color obtained when nitroprusside is added. The same amount of ferricyanide should be required to abolish the nitroprusside test of denatured egg albumin in guanidine hydrochloride solution whether the ferricyanide is added before or 30 minutes after the addition of guanidine hydrochloride. Details of the nitroprusside test and the ferricyanide titration are given in later sections.

Guanidine hydrochloride can be recrystallized from water with a yield of 21 per cent. This recrystallization is used to obtain a good product for comparative purposes, not to prepare the bulk of guanidine hydrochloride used. 20 gm. of guanidine hydrochloride are dissolved in 16 cc. of water at 50°C. The solution is cooled in salt ice water and the guanidine hydrochloride is filtered off in the cold on a pre-cooled Buchner funnel. After the filter cake has been sucked and pressed as dry as possible it is placed in a desiccator.

As much as 5 gm. of guanidine hydrochloride can be dissolved in 1 cc. of water at 100°C. On cooling, however, too thick a suspension of solid is obtained for purification purposes.

Recrystallization from 80 per cent alcohol gives a 55 per cent yield and always improves the product. A completely satisfactory product is obtained from a single recrystallization, however, only if the original amount of impurities is not too great. 20 gm. of guanidine hydrochloride are dissolved in 11.4 cc. of 80 per cent alcohol at 100°C. The solution is brought about as quickly as possible, since guanidine hydrochloride gradually changes into a water-insoluble material at high temperatures. The solution is cooled and filtered as before, washed with cold absolute alcohol, and dried in a vacuum desiccator over NaOH.

Satisfactory guanidine hydrochloride can be prepared from purified guanidine carbonate. Guanidine carbonate is not hygroscopic and it is much less soluble than the hydrochloride.

First, guanidine carbonate (American Cyanamid) is stirred up with twice its weight of water and filtered. If the first part of the filtrate is not perfectly clear it is refiltered. An equal volume of 95 per cent alcohol is added to the filtrate with mechanical stirring. The resulting suspension is cooled to 0°C., filtered in the cold on a Buchner funnel, washed with cold 95 per cent alcohol, and sucked and pressed as dry as possible. Concentrated hydrochloric acid is added to the solid carbonate first with hand stirring and when the suspension becomes fluid with mechanical stirring. When the fizzing on the addition of a drop of acid becomes weak, 1.0 N hydrochloric acid is added to complete the neutralization to green to brom thymol blue. 1 or 2 cc. of water are added to a drop of guanidine hydrochloride solution before the indicator test. The solution should remain green to the indicator even after continued stirring since the carbon dioxide formed is not removed immediately. The solution is allowed to stand in the cold for a few hours, filtered to remove a small amount of brown precipitate, kept at 50°C. in a vacuum oven for 24 hours, and finally dried completely in a vacuum desiccator. During the drying the material is stirred occasionally to break up the caking.

Since guanidine carbonate is not stable indefinitely at 50°C. if the amount of solution being dried is too great to be handled by the vacuum oven in 24 hours, either the solution is evaporated in successive small portions or the solution is first evaporated to a thick suspension on an electric hot plate. The solution heated on the hot plate is placed in a Pyrex Top of the Oven frying pan, is stirred with an L shaped glass stirrer, and a blast

of air from a strong fan is directed on the solution. Under these conditions rapid evaporation takes place without the temperature going above 50°C., or during most of the evaporation above 37°C. This procedure is extraordinarily simple and effective.

The Nitroprusside Test.—The nitroprusside test in guanidine hydrochloride solution is carried out as follows. To 0.5 cc. of 1 or 2 per cent egg albumin there are added 2 drops of neutral 1.0 M phosphate, and 0.7 gm. guanidine hydrochloride. The tube containing the solution is placed in 37°C. water for 2–3 minutes and then in ice water. After the solution has been cooled, there are added 1 drop of 5 per cent sodium nitroprusside and 1 drop of 27 per cent ammonia. 0.5 cc. of 2 per cent egg albumin gives, within a third, as strong a color as 0.5 cc. of 0.002 M cysteine. The conditions for a quantitative nitroprusside test have not been worked out.

A little cyanide can be added to combine with traces of heavy metal compounds. 1 drop of 0.1 N KCN or NaCN is added before the addition of guanidine. No nitroprusside test is obtained in the presence of this small amount of cyanide with cystine or egg albumin whose SH groups have been oxidized to S-S groups by the addition of ferricyanide in guanidine hydrochloride solution, as described in a later section.

The S-S form of egg albumin gives a nitroprusside test when strong cyanide is added which can reduce S-S to SH. To a guanidine hydrochloride solution of the S-S protein, 1 drop of 2 N NaCN and 1 drop of ammonia are added, the solution is allowed to stand 5 minutes at room temperature and is then cooled in ice water. On the addition of 1 drop of nitroprusside a pink color is obtained.

If the nitroprusside test described in the first paragraph of this section is carried out in a solution of denatured egg albumin containing urea instead of guanidine hydrochloride the color obtained is much less intense than if guanidine hydrochloride is used.

If egg albumin is denatured by trichloracetic acid or Duponol PC and no guanidine hydrochloride or urea is present then only a negligible weak pink color is obtained on the addition of nitroprusside and ammonia. If the ammoniacal solution of egg albumin denatured by trichloracetic acid or Duponol PC is saturated with ammonium sulfate, the protein is precipitated and this precipitate gives a faint pink color with nitroprusside. This faint pink color is much intensified on the further addition of solid guanidine hydrochloride or solid thiocyanate.

Titrations in Guanidine Hydrochloride Solution.—First, the standard titration procedure. To 0.5 cc. of 2 per cent egg albumin there are added 0.1 cc. of buffer solution containing equal parts of 1.0 M Na_2HPO_4 and 1.0 M NaH_2PO_4, 0.5 cc. of 0.002 M ferricyanide, tetrathionate, or mercuribenzoate, and 1.2 gm. of guanidine hydrochloride of tested purity. The solution is placed in 37°C. water for 3 minutes and cooled in ice water. Then 1 drop of 5 per cent nitroprusside and 1 drop of 27 per cent ammonia are added. No pink color is observed. If 1 drop of 0.1 N NaCN is added before the nitroprusside, still no pink color is obtained. If 0.5 cc. of 0.0018 M ferricyanide, tetrathionate, or mercuribenzoate is added, then a weak pink is obtained about equal to that obtained from 0.5 cc. of 0.2 per cent albumin to which no SH reagent has been added.

If 0.5 cc. of 0.002 M ferricyanide or mercuribenzoate is used, no nitroprusside test is obtained whether the nitroprusside test is carried out as quickly as possible after the solution of the guanidine and cooling of the solution or after the solution containing protein, ferricyanide, and guanidine has stood 30 minutes at 37°C. If 0.5 cc. of 0.0018 M ferricyanide or mercuribenzoate is used a small nitroprusside test is obtained whether the test is carried out as soon as possible or after 30 minutes.

If the guanidine hydrochloride used is free of impurities which bring about the abolition of SH groups, the ferricyanide can be added 30 minutes after the guanidine hydrochloride without any change in the amount of ferricyanide needed to abolish the nitroprusside test. To 0.5 cc. of protein solution plus 0.1 cc. of phosphate buffer there is added 0.7 gm. of guanidine hydrochloride. The solution is allowed to stand 30 minutes at 37°C., 0.5 cc. of ferricyanide is added, the solution is allowed to stand 3 minutes more at 37°C. before being cooled in ice water. Finally 1 drop of nitroprusside and 1 drop of ammonia are added to find out whether the SH groups have all been oxidized.

The standard titration can be carried out with 0.5 cc. of 0.4 per cent egg albumin instead of 0.5 cc. of 2 per cent egg albumin. The less protein is used, the weaker the nitroprusside test given when only 10 per cent of the SH groups survive. Instead of titrating a dilute solution, one can concentrate the protein. A volume of egg albumin solution containing 10 mg. of protein is diluted to 9 cc. with water and 1 cc. of 2.0 N trichloracetic acid is added. The precipitate is centrifuged down and dissolved with the minimum amount of 0.5 N sodium hydroxide and the resulting solution is diluted to approximately 0.5 cc. with water. Then buffer, titrating agent, and guanidine hydrochloride are added as in the standard procedure.

Formaldehyde cannot be used as a titrating agent because it abolishes the protein SH groups only when added in excess. If 0.5 cc. of 0.004 M formaldehyde is added to the 0.5 cc. of 2 per cent egg albumin before the guanidine under the standard titration conditions, a strong positive nitroprusside is obtained. If 0.5 cc. of 38 per cent formaldehyde is added, only a slight flash of pink is observed.

Effect of Cyanide on Titrations.—The ferricyanide titration cannot be carried out in the presence of cyanide because cyanide in some way inhibits the reduction of ferricyanide by denatured egg albumin, as shown by the following experiment. 1 drop of 0.1 N NaCN is added to the protein solution before the addition of phosphate, ferricyanide, and guanidine. A strong nitroprusside test is obtained although in the absence of cyanide the nitroprusside test would be negative.

Although concentrated ferricyanide oxidizes cyanide slowly, under the conditions of the experiments just described no ferrocyanide is formed from ferricyanide by a cyanide-guanidine hydrochloride-phosphate solution which does not contain protein.

0.5 cc. of 0.002 M free cysteine in a guanidine hydrochloride-phosphate solution reduces ferricyanide in the presence as well as in the absence of 1 drop of 0.1 N NaCN. In the presence of the cyanide, however, the disappearance of the brown ferricyanide color is slow enough to be observed. In the absence of cyanide the disappearance of the brown color takes place instantaneously so far as the eye can tell.

Just as 10 mg. of denatured egg albumin in guanidine hydrochloride solution still gives a nitroprusside test after being treated with 0.5 cc. of 0.002 M ferricyanide in the presence of 1 drop of 0.1 N cyanide, so a strong nitroprusside is also obtained after treatment of 10 mg. denatured egg albumin with 0.5 cc. of 0.002 M mercuribenzoate in the presence of cyanide. Presumably cyanide combines with the heavy metal in mercuribenzoate and so prevents the mercuribenzoate from combining with SH groups. In contrast, 0.5 cc. of 0.002 M tetrathionate abolishes the nitroprusside test in the presence as well as in the absence of 1 drop of 0.1 N cyanide.

Tests for Completeness of SH Reactions in Neutral Guanidine Hydrochloride Solution.—The following series of experiments was designed to find out whether the abolition of the SH groups by the SH reagents takes place entirely in the 3 minute reaction in neutral

solution, or whether part of the abolition of SH groups takes place after ammonia is added for the nitroprusside test.

After the protein and titrating agent have been in the neutral guanidine hydrochloride solution for 3 minutes at 37°C. under the conditions of the standard titration water is added to 9 cc. and then 1 cc. of 2.0 N trichloracetic acid. The precipitate is centrifuged, washed with 0.2 N trichloracetic acid, and centrifuged again. Water is added to the precipitate to make the volume approximately 1 cc. (previously marked on the tube) and the precipitate is dissolved with 1 gm. of guanidine hydrochloride and cooled in ice water. Then nitroprusside and ammonia are added. The protein treated with ferricyanide for 3 minutes gives no nitroprusside test, the protein treated with tetrathionate a moderately strong test, the protein treated with mercuribenzoate about as strong a test as untreated protein. The experiments are repeated, trichloracetic being added after tetrathionate and mercuribenzoate have stood in the neutral protein-guanidine solution for 30 minutes instead of 3 minutes. This time the protein treated with tetrathionate gives no nitroprusside test (even if 1 drop of 0.1 N cyanide is present during the tetrathionate reaction) but the protein treated with mercuribenzoate still gives as strong a test as before. Thus the reaction between ferricyanide and the SH groups of denatured egg albumin in neutral guanidine solution (before the addition of ammonia) is completed in 3 minutes, whereas the reaction with tetrathionate is completed in 30 minutes but not in 3 minutes.

The fact that the trichloracetic acid precipitate of albumin treated with mercuribenzoate gives a nitroprusside test shows that the compound between mercuribenzoate and the protein SH groups must be dissociated by trichloracetic acid, for the following experiment shows that mercuribenzoate actually does combine with the SH groups of denatured egg albumin in neutral solution. After the protein solution containing guanidine hydrochloride and mercuribenzoate has stood 3 minutes, 2 drops of 0.01 M ferricyanide are added. The protein after being precipitated and washed with trichloracetic acid still gives a strong nitroprusside test in guanidine solution. If mercuribenzoate is omitted the nitroprusside test is abolished by ferricyanide. Thus mercuribenzoate prevents the oxidation of the SH groups by ferricyanide in neutral guanidine solution.

Ferricyanide Reduction Test for SH Groups.—The SH groups of the trichloracetic acid precipitate of protein treated with ferricyanide or tetrathionate in guanidine hydrochloride solution can also be measured by the ferricyanide-Duponol PC method. The results confirm those obtained by the nitroprusside test.

The washed trichloracetic acid precipitate is dissolved by the addition of 0.5 cc. of 10 per cent Duponol PC, 0.3 cc. of 0.5 N NaOH (to neutralize the trichloracetic acid), and 0.2 cc. of the neutral phosphate buffer. 0.5 cc. of 0.1 M ferricyanide is added and after the solution has been in a 37°C. bath for 10 minutes the ferrocyanide formed is measured as Prussian blue as described in the following section. The proteins treated with ferricyanide for 3 minutes or with tetrathionate for 30 minutes give no ferrocyanide just as they give no nitroprusside test. The protein treated with tetrathionate for 3 minutes gives the equivalent of 1 cc. of 0.0006 M ferrocyanide just as it gives a moderate nitroprusside test. Protein originally treated with 1 cc. of 0.0008 M ferricyanide gives 1 cc. of 0.00016 M ferrocyanide just as it gives a small nitroprusside test.

The procedure just described can be used to test the effect of cyanide on the oxidation of the SH groups of egg albumin by ferricyanide and tetrathionate. 10 mg. of denatured egg albumin in guanidine hydrochloride solution are exposed for 30 minutes to 0.5 cc.

of 0.002 M ferricyanide or tetrathionate in the presence of 1 drop of 0.1 cyanide. The protein is then precipitated and washed with trichloracetic acid, dissolved in neutral Duponol PC solution, and the surviving SH groups measured with ferricyanide. Although in the absence of cyanide all the SH groups are abolished, in the presence of cyanide ferricyanide abolishes only 55 per cent of the SH groups, tetrathionate 95 per cent. This confirms the result obtained by the nitroprusside test that cyanide interferes with the ferricyanide reaction more than it interferes with the tetrathionate reaction.

Ferricyanide Reduction in Guanidine Hydrochloride Solution.—To 0.5 cc. of 5 per cent egg albumin there are added 2 drops of phosphate buffer, 1 drop of 0.1 or 0.5 M ferricyanide, and 0.6 gm. guanidine hydrochloride. After the solution has been kept in a 37°C. water bath for 3 minutes there are added 1 cc. of water, 0.5 cc. of 2.0 N sulfuric acid, 18 cc. of water, and 2.5 cc. of 2.0 N trichloracetic acid. The suspension is well mixed and centrifuged. If any particles remain in the supernatant solution, the centrifuging is repeated. Filtration results in some loss of ferrocyanide. To 9 cc. of the supernatant solution there are added 0.5 cc. of 0.1 M ferricyanide and 0.5 cc. of ferric sulfate solution. After 20 minutes the Prussian blue is read in the light transmitted by a red filter against the Prussian blue developed from 1 cc. of 0.0025 M ferrocyanide. The two color values agree within 5 per cent. The ferrocyanide standard is made up as follows. To 0.5 cc. of 5 per cent egg albumin there are added 2 drops phosphate solution and 0.6 gm. guanidine hydrochloride. After the solution has stood at 37°C. for 3 minutes 1 cc. of 0.0025 M ferrocyanide is added. Then sulfuric acid and the other reagents are added as before to develop the Prussian blue.

Since guanidine hydrochloride in sufficient concentration interferes with the development of Prussian blue, the experiment is arranged so as to keep the concentration of guanidine hydrochloride as low as possible.

Guanidine hydrochloride, unlike Duponol PC, does not prevent the precipitation of denatured egg albumin by acid ferric sulfate. That is why the protein is precipitated with trichloracetic acid and removed before the addition of ferric sulfate. Duponol PC cannot be added to the acid solution to keep the protein in solution because it forms a precipitate with guanidine hydrochloride.

Ferricyanide Reduction in Urea Solution.—In one test tube there are added to 0.5 cc. of 2 per cent egg albumin 2 drops of 1 N hydrochloric acid and 0.6 gm. urea. After this test tube has been at 37°C. for 5 minutes there is added from another test tube a mixture of 0.5 cc. 0.002 M or 0.1 M ferricyanide, 2 drops 1 N sodium hydroxide, 4 drops 1 M neutral phosphate, and 0.8 gm. urea. After 5 minutes more at 37°C. the reaction is stopped by 0.5 cc. of 2 N sulfuric acid and then there are added 0.5 cc. of 1 M ferricyanide (only to the solution containing 0.5 cc. of 0.002 M ferricyanide), water to 9.5 cc., and 0.5 cc. ferric sulfate solution. The Prussian blue formed is equivalent to 1 cc. of 0.001 M ferrocyanide, within 5 per cent, whether 0.002 M or 0.1 M ferricyanide is originally added.

Titrations in Duponol PC Solution.—First the mercuribenzoate titration. To 1 cc. of 1 per cent egg albumin are added 2.3 cc. of water, 0.2 cc. of the neutral 1.0 M phosphate, and 0.5 cc. of 10 per cent Duponol PC. The solution is brought to 37°C. and 0.5 cc. of 0.02 M ferricyanide is added. After the solution has been at 37°C. for 1 minute there are added 0.5 N of 2.0 N sulfuric acid, water to 9.5 cc., and 0.5 cc. of ferric sulfate solution. After 20 minutes the Prussian blue developed is estimated colorimetrically in the light transmitted by a red filter. The Prussian blue is the same, within 5 per cent, as that developed from 1 cc. of 0.001 M ferrocyanide (*cf.* Anson, 1939). If 1 cc. of 0.001 M

mercuribenzoate is added to the protein-Duponol solution before the ferricyanide and the solution is allowed to stand 1 minute at 37°C. before the addition of ferricyanide, then no Prussian blue is obtained. Thus mercuribenzoate prevents the reduction of ferricyanide by denatured egg albumin in Duponol PC as in guanidine hydrochloride solution. In practice, to avoid the difficulty of estimating very weak colors, 1 cc. of 0.001 M ferrocyanide is added after the reaction has been stopped with sulfuric acid. The Prussian blue obtained is that expected from 1 cc. of 0.001 M ferrocyanide. If 1 cc. of 0.0008 M mercuribenzoate is used instead of 0.001 M mercuribenzoate, then on the addition of 1 cc. of 0.001 M ferrocyanide as much Prussian blue is developed (to quote a single experiment) as would be developed from 1 cc. of 0.00115 M ferrocyanide. The 0.0008 M mercuribenzoate does not completely abolish the SH groups and so some ferricyanide is reduced.

A control experiment is carried out to show that under the conditions used mercuribenzoate does not interfere with the estimation of ferrocyanide. 1 cc. of 0.001 M ferrocyanide is added after the mercuribenzoate and the ferricyanide is added after the sulfuric acid which stops all reduction of ferricyanide. The Prussian blue formed is that expected from the amount of ferrocyanide added. If, however, the solution containing mercuribenzoate and ferrocyanide is allowed to stand 5 minutes (instead of 1 minute as in the actual experiment) before the addition of acid ferric sulfate, then less than the expected amount of Prussian blue is obtained. Presumably in the presence of mercury salt and air some ferrocyanide is oxidized.

Another control experiment shows that under the conditions used mercuribenzoate does not reduce ferricyanide. After the ferricyanide has been allowed to react with denatured egg albumin in the absence of mercuribenzoate, 1 cc. of 0.001 M mercuribenzoate is added and the solution is allowed to stand 1 minute before the addition of acid and ferric sulfate. The amount of Prussian blue obtained is the same as that obtained when mercuribenzoate is not added.

For the ferricyanide and tetrathionate titrations in Duponol PC solution the nitroprusside test is used for the end point. To 0.5 cc. of 2 per cent egg albumin are added 0.2 cc. phosphate solution, 0.5 cc. of 0.002 M ferricyanide or tetrathionate, and 0.5 cc. of 0.8 per cent Duponol PC solution. After this solution has stood 10 minutes in the ferricyanide titration and 30 minutes in the tetrathionate titration water is added to 9 cc., the protein is precipitated by the addition of 1 cc. 2.0 N trichloracetic acid and warming of the solution to 60°C., washed with 0.2 N trichloracetic acid, diluted to 1 cc., and dissolved with 1 gm. of guanidine hydrochloride. No pink color is obtained on the addition of nitroprusside and ammonia. If 10 per cent less ferricyanide or tetrathionate is used, a weak color is obtained in the nitroprusside test.

Only 4 mg. of Duponol PC is used in the experiment just described because larger amounts of Duponol interfere with the precipitation of the protein with trichloracetic acid. 10 mg. of Duponol PC does not prevent the precipitation of the ordinary SH form of egg albumin, but it prevents the precipitation by trichloracetic acid if the protein SH groups are first oxidized to S-S groups.

A control experiment shows that even when only 4 mg. of Duponol PC is used to denature the egg albumin, all the 0.5 cc. of 0.002 M ferricyanide added is reduced to ferrocyanide. After the ferricyanide has reacted with the denatured egg albumin in neutral Duponol solution, there are added 0.5 cc. of 2.0 N sulfuric acid, 0.5 cc. of 10 per cent Duponol PC, 0.5 cc. of 0.1 M ferricyanide, water to 9.5 cc., and 0.5 cc. of the ferric

sulfate solution. As much Prussian blue is formed, within 5 per cent, as from 1 cc. of 0.001 M ferrocyanide. Extra Duponol is added after the reaction has been stopped by sulfuric acid to prevent the precipitation of protein by acid ferric sulfate. Extra ferricyanide is added to speed up the formation of Prussian blue in Duponol solution.

Cyanide inhibits the reduction of ferricyanide by denatured egg albumin in Duponol solution even more than it does in guanidine hydrochloride solution. If in the experiment just described 1 drop of 0.1 N cyanide is added before the addition of Duponol and the ferricyanide is in contact with the albumin 30 minutes, only 1 cc. of 0.00007 M ferrocyanide is formed. In contrast 0.5 cc. of 0.002 M tetrathionate abolishes the nitroprusside test of 10 mg. of denatured albumin even if 1 drop of 0.1 N cyanide is present during the tetrathionate reaction.

Reactions of Iodine with Native Egg Albumin.—First, the experiments showing the minimum amount of iodine which abolishes the nitroprusside test. To 0.5 cc. of 2 per cent egg albumin are added at 0°C. 0.1 cc. of neutral 1.0 M phosphate and 0.5 cc.of 0.0026 N iodine. The solution is allowed to stand 5 minutes at 0°C., during which time all the iodine added is absorbed as shown by a negative starch test. The egg albumin treated with iodine whether dialyzed free of iodide or not gives a negative nitroprusside test in guanidine hydrochloride solution but if it is first allowed to stand in an alkaline solution containing strong cyanide, a strong positive nitroprusside test is obtained. The techniques of the nitroprusside tests are described in the section on the nitroprusside test. If 10 per cent less iodine is used a weak positive nitroprusside test is obtained without the preliminary treatment with alkaline cyanide.

In the next experiments more iodine is added at 37°C., and there are no surviving S-S groups which give the nitroprusside test after exposure to alkaline cyanide and no uniodinated tyrosine groups which give the Millon test. To 0.5 cc. of 2 per cent egg albumin there are added 0.1 cc. of phosphate buffer and 0.5 cc. of 0.08 N iodine. The solution is allowed to stand 2 hours at 37°C. Then water is added to 9 cc. and 1 cc. of 2.0 N trichloracetic acid. The precipitate is centrifuged, stirred up with 10 cc. of 0.2 N trichloracetic acid, and centrifuged again. The precipitate is suspended in enough water to make the volume approximately 1 cc., and is dissolved with 1.2 gm. guanidine hydrochloride, and 1 drop of 2.0 N cyanide and 1 drop of ammonia are added. After 5 minutes 1 drop of 5 per cent nitroprusside is added. No pink color is obtained. A Millon test is carried out on the 0.5 cc. of the precipitate washed with trichloracetic acid by adding 3 drops of Millon's reagent and heating in boiling water for 2–3 minutes. No purple color is obtained. The Millon reagent used is made up as follows. 10 gm. of mercury are digested in 20 gm. of nitric acid of specific gravity 1.42 until NO_2 no longer comes off. The solution is diluted with twice its volume of water and stored in a brown bottle. In carrying out the Millon test it is necessary to heat long enough to bring out the full color and not long enough to make the color disappear again.

If 0.06 N iodine is added to 37°C. in the experiment just described, instead of 0.08 N iodine, the protein after being precipitated with trichloracetic acid gives a weak cyanide-nitroprusside test and a weak Millon test.

If after the iodine is added the solution is allowed to stand 1 hour at 60°C. instead of 2 hours at 37°C., 0.5 cc. of 0.05 N iodine has to be added to abolish the cyanide-nitroprusside and the Millon test of 0.5 cc. of 2 per cent egg albumin. If 0.04 N iodine is added faint positive tests are obtained.

A control experiment is done to show that the trichloracetic precipitation and washing adequately removes the tetrathionate formed by the reaction of iodine and thiosulfate.

0.5 cc. of 0.08 N iodine and 0.5 cc. 0.08 M thiosulfate are mixed before being added to egg albumin. The protein after being precipitated and washed with trichloracetic acid gives strongly positive cyanide-nitroprusside and a strongly positive Millon test.

Mercuribenzoate Plus Native and Denatured Egg Albumin.—The following experiments show that if mercuribenzoate combines with native egg albumin at all, the compound is much looser than the compound between mercuribenzoate and denatured egg albumin.

First it is shown that under the conditions used ferricyanide is not reduced by either native egg albumin or by denatured egg albumin in Duponol solution. To 1 cc. of 1 per cent native egg albumin are added 0.2 cc. of phosphate buffer and 0.5 cc. of water. The solution is cooled to 0°C. 1 cc. of 0.001 M ferricyanide previously cooled to 0°C. is added, the solution is allowed to stand 1 minute at 0°C., and then there are added 0.5 cc. of 2.0 N sulfuric acid, 1 cc. of 0.001 M ferrocyanide, 0.5 cc. of 0.02 M ferricyanide, 0.5 cc. of 10 per cent Duponol PC, water to 9.5 cc., and 0.5 cc. ferric sulfate solution. The Prussian blue obtained is the same in amount as the Prussian blue obtained from 1 cc. of 0.001 M ferrocyanide alone, indicating no reduction of ferricyanide by native egg albumin.

The same experiment is repeated with denatured egg albumin, 0.5 cc. of 10 per cent Duponol PC being added to 1 cc. of egg albumin instead of 0.5 cc. of water and no Duponol being added after the acid. Again no ferricyanide is reduced.

The next experiments show that cysteine added to native or denatured egg albumin is free to reduce ferricyanide. 1 cc. of cold 0.001 N cysteine (in 0.01 N hydrochloric acid) is added to the cold native or denatured egg albumin, the ferricyanide added as promptly as possible after the cysteine, and the acid is added 1 minute later. The equivalent of 0.75 – 0.9 cc. of ferrocyanide is formed. Some cysteine is unavoidably oxidized by the oxygen of the air and the amount oxidized is variable.

Finally, the experiments which show whether or not added mercuribenzoate is free to combine with cysteine. 1 cc. of 0.001 M mercuribenzoate is added to the native or denatured egg albumin before the solution is cooled and cysteine and ferricyanide are added. In the solution of native egg albumin no ferrocyanide is formed, showing that the mercuribenzoate has combined with the cysteine and that the cysteine-mercuribenzoate compound does not reduce ferricyanide. In the solution of denatured egg albumin, however, the same amount of ferrocyanide is formed from ferricyanide as in the absence of mercuribenzoate, showing that the mercuribenzoate has combined with the protein and is not removed from the protein by the cysteine which remains free to reduce ferricyanide.

If the experiment in Duponol solution is repeated with 1 cc. of water substituted for the 1 cc. of protein solution, no ferrocyanide is formed, showing that mercuribenzoate can combine with the cysteine in the Duponol solution if the mercuribenzoate is not combined with protein.

The Mercuribenzoate-Cysteine Compound.—If 1 cc. of 0.001 M cysteine is added to a neutral phosphate solution containing 1 cc. of 0.0001 M mercuribenzoate, the resulting solution does not give a nitroprusside test but does immediately decolorize 1 cc. of 0.001 N iodine solution.

SUMMARY

1. 1 cc. of 0.001 M ferricyanide, tetrathionate, or *p*-chloromercuribenzoate is required to abolish the SH groups of 10 mg. of denatured egg albumin in

guanidine hydrochloride or Duponol PC solution. Both the nitroprusside test and the ferricyanide reduction test are used to show that the SH groups have been abolished.

2. 1 cc. of 0.001 M ferrocyanide is formed when ferricyanide is added to 10 mg. of denatured egg albumin in neutral guanidine hydrochloride or urea solution. The amount of ferricyanide reduced to ferrocyanide by the SH groups of the denatured egg albumin is, within wide limits, independent of the ferricyanide concentration.

3. Ferricyanide and *p*-chloromercuribenzoate react more rapidly than tetrathionate with the SH groups of denatured egg albumin in both guanidine hydrochloride solution and in Duponol PC solution.

4. Cyanide inhibits the oxidation of the SH groups of denatured egg albumin by ferricyanide.

5. Some samples of guanidine hydrochloride contain impurities which bring about the abolition of SH groups of denatured egg albumin and so interfere with the SH titration and the nitroprusside test. This interference can be diminished by using especially purified guanidine hydrochloride, adding the titrating agent before the protein has been allowed to stand in guanidine hydrochloride solution, and carrying out the nitroprusside test in the presence of a small amount of cyanide.

6. The SH groups of egg albumin can be abolished by reaction of the native form of the protein with iodine. It is possible to oxidize all the SH groups with iodine without oxidizing many of the SH groups beyond the S-S stage and without converting many tyrosine groups into di-iodotyrosine groups.

7. *p*-chloromercuribenzoate combines with native egg albumin either not at all or much more loosely than it combines with the SH groups of denatured egg albumin or of cysteine.

8. The compound of mercuribenzoate and SH, like the compound of aldehyde and SH and like the SH in native egg albumin, does not give a nitroprusside test or reduce ferricyanide but does reduce iodine.

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