THE REACTIONS OF DENATURED EGG ALBUMIN WITH FERRICYANIDE*

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INTRODUCTION

This paper describes the reactions between the reducing groups of denatured egg albumin and ferricyanide. Measurements have been made of the effects on the amount of ferricyanide reduced of varying the time, temperature, and pH of the reaction, and the concentration of ferricyanide; of adding the synthetic detergent, Duponol PC; and of treating the denatured protein before the ferricyanide reaction with formaldehyde and iodoacetamide, which are known to react with SH groups. The main result is that, provided the ferricyanide concentration is not too high and Duponol PC is present, there is a definite reaction between ferricyanide and denatured egg albumin. By a definite ferricyanide reaction is meant one in which the amount of ferricyanide reduced is, within wide limits, independent of the time, temperature, and pH of the reaction, and of the concentration of ferricyanide.

For the experiments on protein denaturation for which the definite reaction with ferricyanide was worked out and for many other practical applications of this reaction it is not necessary to know what protein groups reduce dilute ferricyanide in Duponol PC solution or how Duponol PC influences the reaction. The reactions between ferricyanide and various amino acids and proteins which will be described, however, indicate that the new definite reaction between ferricyanide and denatured egg albumin is a reaction with SH groups and that the effect of Duponol PC is to lower the ferricyanide concentration at which the SH groups of denatured egg albumin react with ferricyanide.

* A brief account of the reactions of denatured egg albumin with ferricyanide and of native egg albumin with iodine and iodoacetamide has been published in *Science* (Anson, 1939*a*).

HISTORICAL

The SH groups of denatured egg albumin have usually been studied by adding an oxidizing agent and measuring how much of the oxidizing agent is reduced. Thus it was found that 10 mg. of denatured egg albumin reduces about 0.0005 milliequivalents of cystine (Mirsky and Anson, 1935) and porphyrindin (Kuhn and Desnuelle, 1938; Greenstein, 1938); that is, the amounts of cystine and porphyrindin which would be reduced by 0.0005 mM of cysteine. Conditions were chosen under which all the SH groups and no other protein reducing groups were supposedly oxidized.

Mirsky and Anson also studied the SH groups of denatured egg albumin by an indirect method. Egg albumin was hydrolyzed with sulfuric acid and 0.0005 mM of cysteine was found in the hydrolysate of 10 mg. of albumin. If before the hydrolysis, the egg albumin was denatured, the denatured protein treated with ferricyanide or iodoacetate, and the excess ferricyanide or iodoacetate removed, then no cysteine was found on hydrolysis. Two conclusions were drawn from these results, that all the SH groups found on hydrolysis are free and reactive in denatured but unhydrolyzed egg albumin, and that all the free and reactive SH groups of denatured egg albumin are oxidized by cystine.

Greenstein found that denatured egg albumin in the presence of guanidine hydrochloride reduces about twice as much porphyrindin as heat denatured egg albumin in the absence of guanidine. The conclusion was drawn from this result that guanidine creates new SH groups in denatured egg albumin by breaking some as yet unidentified SH linkage.

Greenstein found that urea increases the amount of porphyrindin reduced by denatured egg albumin almost as much as guanidine. Although the results have never been published, I have used urea for many years to promote the reaction between protein reducing groups and the uric acid reagent.

Mirsky and Anson (1936) studied the reduction of ferricyanide by denatured egg albumin at pH 9.6. The amount of ferrocyanide formed was greater the higher the concentration of ferricyanide and the temperature and the longer the time of reaction. Ferricyanide was reduced even if the SH groups of denatured egg albumin were first oxidized by cystine. At pH 9.6 ferricyanide was found to oxidize not only cysteine but also tyrosine and tryptophane. The amount of ferricyanide reduced by tyrosine and tryptophane was greater the higher the concentration of ferricyanide and the temperature and the longer the time of reaction. It is known that in the oxidation of tyrosine a whole series of oxidation products can

be formed. From these results it was concluded that at pH 9.6 ferricyanide oxidizes tyrosine and tryptophane reducing groups of denatured egg albumin as well as SH groups.

The hydrolysate of 10 mg. of egg albumin contains 0.0015 mM of SH plus S—S sulfur. This value has been obtained by Kassell and Brand (1938) using their modification of Baernstein's method, and by S. Graff[1] using the Graff, Maculla, and Graff (1937) modification of the method of Vickery and White.

The Reactions between Denatured Egg Albumin and Ferricyanide

I have found that at pH 6.8 as at pH 9.6 the amount of ferricyanide reduced by denatured egg albumin is greater the higher the concentration

TABLE I

Reactions between Ferricyanide and 10 Mg. Denatured Egg Albumin

How denatured	Ferricyanide	Time	Temperature	pH	Ferrocyanide formed
	mM	*min.*	*°C.*		*mM*
HCl	0.002	10	37	6.8	0.00064
HCl	0.002	60	37	6.8	0.00075
HCl	0.05	10	37	6.8	0.00094
HCl	0.5	10	37	6.8	0.001
HCl	0.5	60	37	6.8	0.0012
HCl	0.002	10	37	9.6	0.00074
HCl	0.05	10	37	9.6	0.00123
Cl_3COOH	0.002	10	37	6.8	0.00063
Cl_3COOH	0.5	10	37	6.8	0.0011

of ferricyanide and the temperature and the longer the time of reaction (Table I). The difference between the results at pH 6.8 and at 9.6 is that a given amount of ferrocyanide is formed at a lower concentration of ferricyanide at pH 9.6 than at pH 6.8.

The synthetic detergent, Duponol PC (Du Pont), has a great effect on the reaction between denatured egg albumin and ferricyanide. Duponol PC denatures proteins such as hemoglobin and egg albumin and keeps the denatured protein in solution even at the isoelectric point. Whereas 8 M urea denatures neutral hemoglobin slowly, 0.0008 M Duponol PC denatures neutral hemoglobin rapidly (Anson, 1939*b*). Duponol PC is a more effective denaturant and solvent than most of the detergents I have tried and unlike many other detergents it does not reduce ferricyanide.

[1] Personal communication.

Since it is used in pharmaceutical products Duponol PC is the most carefully prepared of the Duponols.

Table II shows the amounts of ferrocyanide formed when ferricyanide is added under different conditions to egg albumin denatured by Duponol PC.

TABLE II

Reactions between Ferricyanide and 10 Mg. Denatured Egg Albumin in Duponol PC Solution

Ferricyanide	Time	Temperature	pH	Duponol PC	Ferrocyanide formed
mM	*min.*	°C.		*mg.*	*mM*
0.001	2	37	6.8	50	0.00082
0.001	10	37	6.8	50	0.00096
0.001	60	37	6.8	50	0.00098
0.001	10	37	9.6	50	0.00096
0.002	2	37	6.3	10	0.00093
0.002	10	37	6.3	10	0.00099
0.002	2	0	6.8	10	0.00055
0.002	10	0	6.8	10	0.00080
0.002	100	0	6.8	10	0.00095
0.002	2	37	6.8	10	0.00092
0.002	10	37	6.8	10	0.00096
0.002	100	37	6.8	10	0.00098
0.002	2	37	6.8	50	0.00097
0.002	10	37	6.8	50	0.00098
0.002	100	37	6.8	50	0.00105
0.002	2	37	9.6	50	0.00095
0.002	10	37	9.6	50	0.00099
0.002	2	100	6.8	50	0.00095
0.002	10	100	6.8	50	0.00100
0.05	2	37	6.8	10	0.00094
0.05	10	37	6.8	10	0.00096
0.05	10	37	6.8	50	0.00098
0.05	10	37	9.6	10	0.00125
0.2	60	37	6.8	10	0.00105
0.2	20	60	6.8	10	0.00124
0.2	2	100	6.8	10	0.00110
0.2	10	100	6.8	10	0.00136
0.2	20	100	6.8	10	0.00150

First, 0.001 mM of ferrocyanide is formed from 0.001 mM of ferricyanide whereas in the absence of Duponol PC only 0.00064 mM of ferrocyanide is formed at pH 6.8 from 10 mg. of denatured egg albumin and 0.002 mM of ferricyanide (Table I). Thus Duponol PC increases the amount of ferrocyanide formed from ferricyanide in dilute solution.

Secondly, if the ferricyanide concentration is not too high, then 0.001

mM of ferrocyanide is formed despite wide variations in the concentrations of ferricyanide and Duponol PC, the temperature, the time of reaction, and the pH. Thus in the presence but not in the absence of Duponol PC there is a definite reaction between denatured egg albumin and dilute ferricyanide.

Finally, if enough ferricyanide is added, then more than 0.001 mM of ferrocyanide is formed and the amount of ferrocyanide formed is greater, the higher the concentration of ferricyanide and the temperature and the longer the time of reaction. Thus in the presence as well as in the absence of Duponol PC there is an indefinite reaction between denatured egg albumin and ferricyanide, but in the case of egg albumin Duponol PC makes possible a separation between the definite reaction with dilute ferricyanide and the indefinite reaction with concentrated ferricyanide.

At pH 9.6 the results are qualitatively the same as at pH 6.8. At both pH's there is a definite reaction with dilute ferricyanide and an indefinite reaction with more concentrated ferricyanide. At pH 9.6, however, the indefinite reaction begins at a lower ferricyanide concentration than at pH 6.8.

Formaldehyde and iodoacetamide are known to react with SH groups. If denatured egg albumin is first treated with formaldehyde or iodoacetamide, and dilute ferricyanide is then added in neutral Duponol solution, no ferrocyanide is formed.

Reactions of Ferricyanide with Amino Acids and Cysteine-Free Proteins

Before discussing the question of what protein groups react with ferricyanide I shall summarize what is known from previous experiments and from new experiments about the reactions of ferricyanide under various conditions with amino acids and with proteins which do not contain cysteine. The results of the new experiments are given in Table III.

Ferricyanide in neutral solution oxidizes to S—S the SH of glutathione. One molecule of ferrocyanide is formed for each SH group which is oxidized. The ferrocyanide formed can be estimated as Prussian blue (Mason, 1930).

Similarly ferricyanide oxidizes cysteine to cystine. As shown in Table III this oxidation of cysteine takes place even at pH 3.2. Mason found the oxidation of glutathione at pH 3.2 to be slow and incomplete. Thus the ease with which SH groups are oxidized depends on the structure of the whole molecule. Similarly iodoacetate reacts more readily with some SH compounds than with others (Michaelis and Schubert, 1934; Smythe, 1936).

In neutral solution containing Duponol PC, ferricyanide, in dilute solu-

tion, does not react with cystine, tyrosine, tryptophane, or with proteins such as serum albumin, pepsin, and chymotrypsinogen which do not con-

TABLE III

Reactions between Ferricyanide and Amino Acids and Cysteine-Free Proteins

Amino acid or protein	Ferricyanide	Time	Temperature	pH	Duponol PC	Ferrocyanide formed
	mM	*min.*	°C.		*mg.*	*mM*
0.001 mM cysteine	0.002	10	37	1.0	0	0.00018
0.001 mM cysteine	0.002	10	37	1.0	50	0.00016
0.001 mM cysteine	0.002	10	37	2.0	0	0.00023
0.001 mM cysteine	0.002	10	37	2.0	50	0.00025
0.001 mM cysteine	0.002	10	37	3.0	0	0.00098
0.001 mM cysteine	0.002	10	37	3.0	50	0.00092
0.001 mM cysteine	0.002	10	37	6.8	0	0.00098
0.001 mM cysteine	0.002	10	37	9.6	0	0.00101
0.001 mM cysteine	0.002	10	37	9.6	50	0.00101
0.001 mM cysteine	0.05	10	37	9.6	50	0.00104
0.001 mM cysteine	0.2	20	50	6.8	10	0.00104
0.001 mM cysteine	0.2	20	80	6.8	10	0.00149
0.001 mM cysteine	0.2	10	37	9.6	50	0.00144
0.0005 mM cystine	0.01	10	37	9.6	50	0.0
0.0005 mM cystine	0.1	10	37	9.6	50	<0.00005
0.0005 mM cystine	0.2	20	50	6.8	10	0.0001
0.0005 mM cystine	0.2	20	80	6.8	10	0.00062
0.0005 mM cystine	0.2	20	100	6.8	10	0.00144
0.0005 mM cystine	0.5	20	80	6.8	10	0.00093
1 mg. tyrosine	0.002	10	37	6.8	25	0.0
1 mg. tyrosine	0.02	10	100	6.8	25	<0.0001
1 mg. tyrosine	0.1	10	100	6.8	50	0.0009
1 mg. tyrosine	0.2	10	37	6.8	50	<0.0001
0.001 mM tyrosine	0.002	10	37	9.6	50	<0.00005
0.001 mM tyrosine	0.01	10	37	9.6	50	0.00116
0.001 mM tyrosine	0.1	10	37	9.6	50	0.00175
0.001 mM tryptophane	0.01	10	37	6.8	0	0.0
0.001 mM tryptophane	0.1	10	37	6.8	0	<0.00005
0.001 mM tryptophane	0.1	10	70	6.8	0	<0.00005
0.001 mM tryptophane	0.5	10	37	6.8	0	0.0002
20 mg. serum albumin	0.003	10	37	6.8	50	0.0
20 mg. serum albumin	0.2	10	100	6.8	50	0.004
10 mg. pepsin	0.003	10	37	6.8	50	0.0
10 mg. chymotrypsinogen	0.002	10	37	6.8	50	0.0
10 mg. chymotrypsinogen	0.2	20	37	6.8	50	<0.00005
10 mg. chymotrypsinogen	0.2	20	50	6.8	10	0.00038
10 mg. chymotrypsinogen	0.2	20	100	6.8	10	0.00168

tain cysteine. At pH 9.6 the reactions with dilute ferricyanide are very slight.

Concentrated ferricyanide, however, oxidizes cystine, tyrosine, tryptophane, serum albumin, and chymotrypsinogen. The amount of ferrocyanide formed is greater the higher the temperature and the concentration of ferricyanide. At pH 9.6 the reactions begin at a lower ferricyanide concentration than at pH 6.8.

Thus cysteine is the only amino acid which is known to give a definite stoichiometric reaction with ferricyanide and which is known to react with dilute neutral ferricyanide at all. In the few cases which have been studied, proteins which do not contain cysteine do not react with dilute ferricyanide in neutral Duponol PC solution. If the concentration of ferricyanide is high enough there is an indefinite reaction of ferricyanide with cystine, tyrosine, tryptophane, and with proteins which contain these other amino acids but not cysteine. The indefinite reaction begins at a lower ferricyanide concentration at pH 9.6 than at pH 6.8.

The reaction, if any, of the carbohydrate component of egg albumin (Neuberger, 1938) with ferricyanide in Duponol solution has not been studied.

DISCUSSION

The evidence that the definite reaction between dilute ferricyanide and denatured egg albumin in neutral Duponol PC solution is due solely to the oxidation of SH to S—S is as follows:

1. Dilute ferricyanide in neutral solution oxidizes cysteine to cystine. This reaction, like the definite reaction between dilute ferricyanide and denatured egg albumin in Duponol PC solution, is within wide limits independent of the concentration of ferricyanide and the pH.

2. Cystine, tyrosine, and tryptophane and proteins containing these amino acids, but not cysteine, do not react with dilute ferricyanide in Duponol PC solution.

3. Under those conditions under which ferricyanide does react with cystine, tyrosine, tryptophane, and cysteine-free proteins, the reaction is not a definite reaction.

4. Formaldehyde and iodoacetamide abolish the reaction of denatured egg albumin with dilute ferricyanide. These two reagents are not known to react with any reducing groups other than SH groups.

Very little is known about the effects of protein structure on the properties of amino acid groups. It is possible that there are some peculiarly reactive cystine, tyrosine, or tryptophane groups in denatured egg albumin. Very little is known about the oxidation of amino acid reducing groups in complex systems which contain different types of reducing groups.

It is possible that there are in denatured egg albumin cystine, tyrosine, or tryptophane groups which are oxidized by dilute ferricyanide in the presence but not in the absence of SH groups.

The sulfuric acid hydrolysate of 10 mg. of egg albumin was found to contain 0.0005 mM of cysteine (Mirsky and Anson, 1935). Some cysteine was probably lost by oxidation, decomposition, and adsorption by humin. If future experiments, however, show that 10 mg. of egg albumin contains only 0.0005 mM of cysteine then one of three assumptions must be made to explain the further fact that 10 mg. of egg albumin reduces 0.001 mM of dilute ferricyanide in Duponol PC solution. The reduction of ferricyanide may not be due solely to SH groups; new SH groups may be produced by Duponol PC which do not exist in native egg albumin and are not liberated by acid hydrolysis; or there may be an equilibrium between free SH groups and linked SH groups and when the free SH groups are oxidized more free SH groups are formed to maintain the equilibrium.

Thus, all the present experiments are in harmony with the simple conclusion that the oxidation of denatured egg albumin in Duponol PC solution by dilute ferricyanide is due to the oxidation of SH to S—S groups. Other and more complex mechanisms for the reduction of ferricyanide are, however, conceivable. All these conceivable mechanisms cannot be definitely excluded so long as only the formation of ferrocyanide is measured.

The evidence that there are in denatured egg albumin free SH groups which react with concentrated ferricyanide and iodoacetamide in the absence of Duponol PC but not with dilute ferricyanide and that therefore the effect of Duponol PC is merely to lower the concentration of ferricyanide at which ferricyanide reacts with the SH groups of denatured egg albumin is as follows:

1. The amount of ferricyanide reduced by denatured egg albumin in the absence of Duponol PC solution is greater the higher the concentration of ferricyanide, even when the ferricyanide is so dilute that it does not react with cystine, tyrosine, or tryptophane.

2. Denatured egg albumin treated with iodoacetamide in the absence of Duponol PC no longer reduces dilute ferricyanide in the presence of Duponol PC.

The evidence that the indefinite reaction between concentrated ferricyanide and denatured egg albumin in Duponol PC solution is a reaction with cystine, tyrosine, or tryptophane groups is as follows:

1. In the indefinite reaction between concentrated ferricyanide and denatured egg albumin as in the indefinite reaction between concentrated ferricyanide and cystine, tyrosine, tryptophane, and cysteine-free pro-

teins, more ferrocyanide is formed the higher the concentration of ferricyanide and the temperature and the more alkaline the solution.

2. The indefinite reaction between concentrated ferricyanide and denatured egg albumin begins under roughly the same conditions as the indefinite reaction between concentrated ferricyanide and denatured serum albumin and chymotrypsinogen. In both cases, the indefinite reaction begins at a lower ferricyanide concentration at pH 9.6 than at pH 6.8.

Altogether the facts about the reactions of ferricyanide and denatured egg albumin are now in clear and useful form and they have been described quite separately from the conclusions which provide at present the simplest explanation of the facts. It cannot be said that the conclusions have been definitely proven. There is no reagent available which one can be sure reacts with all the SH groups of denatured egg albumin and with no other groups. More certain conclusions will be possible only when some specific reagent is discovered or when the reducing groups of many different proteins are studied more extensively by a number of entirely independent methods.

Greenstein's conclusion that guanidine creates new SH groups by breaking some SH linkage was based on the observation that guanidine increases the porphyrindin titration and on the assumption that even in the absence of guanidine, all the free SH groups of denatured egg albumin are oxidized in the porphyrindin titration. No experimental evidence was given in support of this assumption. The titrations were carried out under only one arbitrarily chosen set of conditions. The concentration of porphyrindin, the time, temperature, and pH of the reaction were not varied. Indeed the instability of porphyrindin sets definite limits to the conditions under which porphyrindin can be used. Furthermore, it is difficult to estimate small amounts of reduced porphyrindin in the presence of large amounts of oxidized porphyrindin.

The ferricyanide experiments suggest by analogy that there are free SH groups in denatured egg albumin which do not react rapidly with the dilute porphyrindin used by Greenstein but which would react with more concentrated porphyrindin and that the effect of guanidine is not to create new SH groups by the breaking of hypothetical SH linkages but to lower the concentration of porphyrindin at which porphyrindin reacts rapidly with the SH groups of denatured egg albumin.

It should be emphasized that there is no contradiction between the porphyrindin experiments themselves and the more varied experiments with ferricyanide at different concentrations and with iodoacetamide. The difference between Greenstein's conclusion and the conclusion sug-

gested by the experiments with ferricyanide and iodoacetamide is a difference in the interpretation of how guanidine and Duponol PC influence the reducing power of denatured egg albumin.

EXPERIMENTAL

Reagents

Egg albumin is crystallized with ammonium sulfate, recrystallized three times, dialyzed against cold distilled water in a shaking dialyzer, and stored frozen. The nitrogen content of the albumin solution is estimated by the Kjeldahl method.

Acid ferric sulfate containing phosphoric acid and gum ghatti is prepared according to Folin and Malmros (1929).

Potassium ferricyanide as obtained commercially always contains some ferrocyanide and some commercial preparations contain a blue impurity. The ferrocyanide in a 0.5 M solution of Merck's reagent potassium ferricyanide is oxidized with just not enough bromine water to oxidize all the ferrocyanide. It is possible to obtain a preparation of ferricyanide treated with bromine such that the addition of 0.5 cc. ferric sulfate solution to 5 cc. of 0.5 M ferricyanide treated with bromine gives an increase in light absorption due to the formation of Prussian blue which is detectable with the photoelectric colorimeter but which is 5 times less than the increase obtained when 1 drop 0.001 M ferrocyanide is added before the ferric sulfate. The ferricyanide is stored at 5°C. in the dark and its ferrocyanide content occasionally checked.

Iodoacetamide.—50 gm. of chloracetamide (Eastman) and 80 gm. of NaI are dissolved in 1 liter of acetone with gentle warming. The solution is allowed to stand 2 days at room temperature, the precipitate of NaCl is filtered off, and the acetone is boiled off *in vacuo*. When the temperature begins to rise rapidly, the evaporation is stopped, and the solution is cooled in ice water. The crystals which are formed are filtered off in the cold and washed with ice cold acetone. The same process of evaporation, etc. is repeated twice more with the filtrates and the washings. Thus decomposition of the iodoacetamide by hot acetone is avoided and an acetone washed product is obtained without too much loss.

The crystals are dried with a current of air and added to an equal weight of water which is warmed rapidly until the crystals are dissolved. The solution is then promptly cooled with ice water and the crystals filtered off in the cold. The crystals are dried in a desiccator and the recrystallization repeated.

Iodoacetamide prepared according to the above directions gives no iodine test with starch and no precipitate with acid silver nitrate, which indicates the absence of iodide and of iodoacetic acid which slowly liberates iodide in acid solution. Iodoacetamide is slowly converted into iodoacetic acid in neutral and alkaline solution.

Duponol.—The Duponols (Du Pont) have the general composition $CH_3(CH_2)_n$-CH_2OSO_3Na. Duponol Special WA Paste consists mainly of the C_{12} compound which is called sodium dodecyl sulfate. The dried form of Special WA Paste is called Duponol ME Dry. The "sodium dodecyl sulfate" used in the present experiments was a dried product kindly provided by Proctor and Gamble. It is not available commercially. Duponol WA Paste is a mixture of the C_{10}-C_{18} compounds and contains small amounts of the free alcohols and Na_2SO_4. Duponol PC is a dried and purified form of Duponol WA Paste from which the free alcohols have been extracted with acetone.

Duponol PC and sodium dodecyl sulfate can be kept as 10 per cent stock solutions.

Buffers.—Sodium buffer salts are used rather than potassium salts which precipitate Duponol. Potassium ferricyanide is used rather than sodium ferricyanide only because satisfactory sodium ferricyanide is not available commercially. Potassium ferricyanide precipitates Duponol PC if more than 0.2 mM is used in the experiments to be described.

The 1.0 M pH 6.8 phosphate buffer is made up from equal parts 1 M Na_2HPO_4 and NaH_2PO_4. The pH 6.3 buffer is made up of 1 part 1 M Na_2HPO_4 and 3 parts 1 M NaH_2PO_4.

The pH 9.6 borate buffer is made up from 7 parts 1 M sodium borate and 3 parts 1 M NaOH.

The Reactions between Ferricyanide and Denatured Egg Albumin

Table I gives the amounts of ferrocyanide formed when ferricyanide is added under various conditions to egg albumin denatured by hydrochloric or trichloracetic acid.

I shall first describe the experiments with egg albumin which has been denatured by HCl and then brought to pH 6.8.

1 cc. of 1 N HCl is added to 4 cc. of 3 per cent dialyzed egg albumin. The test tube containing the solution is placed in 50°C. water for 2 minutes and then is cooled. The solution is neutralized with NaOH to green to brom thymol blue and then diluted to 12 cc.

To 1 cc. of the diluted solution containing 10 mg. of denatured egg albumin are added 0.1 cc. of pH 6.8 phosphate buffer and 1 cc. of ferricyanide solution. After the ferricyanide reaction, the solution is diluted to 9 cc. and mixed with 1 cc. of 2 N trichloracetic acid. The precipitate is filtered off. To 5 cc. of the filtrate is added 0.5 cc. of water, or if the amount of ferricyanide present is less than 0.05 mM 0.5 cc. of a ferricyanide solution containing enough ferricyanide to make the final amount 0.05 mM. Finally Prussian blue is developed by the addition of 0.3 cc. of the ferric sulfate solution and the color is read after 20 minutes against a ferrocyanide standard or against a blue glass calibrated with a ferrocyanide standard. A red color filter is used because red light is strongly absorbed by Prussian blue and weakly absorbed by ferricyanide.

The ferrocyanide standard is made up by adding ferrocyanide to neutralized egg albumin, adding trichloracetic acid, and adding before filtration the same amount of ferricyanide used in the experiment. Ferric sulfate is added to the filtrate as already described.

Although ferricyanide increases the rate at which Prussian blue is formed (why, I do not know) it does not affect significantly the amount of red light absorbed until the amount of ferricyanide is 0.2 mM or more. The amount of Prussian blue formed is the same whether the albumin, trichloracetic acid, and filtration are omitted or not, provided there is no impurity of ferric salts in the reagents. Any Prussian blue which is formed from ferric salts before filtration is removed by filtration.

The experiments at pH 9.6 are carried out exactly as the experiments at pH 6.8 with two exceptions. Before the acid denatured albumin is diluted to 12 cc. it is made pink to phenolphthalein instead of green to brom thymol blue. And 0.3 cc. of borate buffer is added instead of 0.1 cc. of phosphate buffer.

When the egg albumin is denatured by trichloracetic acid instead of by hydrochloric acid 1 cc. of 2 N trichloracetic acid is added instead of 1 cc. of 1 N hydrochloric acid. The suspension is allowed to stand 5 minutes at room temperature and is then neutralized. Otherwise the procedure is exactly that already described.

Table II gives the amounts of ferrocyanide formed when ferricyanide is added under various conditions to denatured egg albumin in the presence of Duponol PC. A few experiments carried out with sodium dodecyl sulfate gave exactly the same results as the experiments with Duponol PC.

I shall first describe the experiments at pH 6.8 and pH 6.3. 0.5 cc. of Duponol PC solution is added to 1 cc. of 1 per cent dialyzed egg albumin. After 5 minutes, 0.2 cc. of phosphate buffer and 1 cc. of ferricyanide are added. After the ferricyanide reaction there are added 0.5 cc. 2 N H_2SO_4, enough Duponol PC solution to make the final amount of Duponol 50 mg., enough ferricyanide to make the final amount 0.05 mM, water to 9.5 cc., and finally 0.5 cc. of ferric sulfate to develop the Prussian blue. Precipitation of the protein by ferric sulfate is prevented by the 50 mg. of Duponol PC.

The ferrocyanide standard is made up in the presence of the amounts of albumin, Duponol PC, sulfuric acid, and ferricyanide used in the experiment. Albumin, Duponol, and ferricyanide in amounts less than 0.2 mM have little effect on the Prussian blue color. The ferricyanide is added after the solution has been acidified with sulfuric acid.

When 0.2 mM or more of ferricyanide is present and the temperature is 80°C. or higher, a small amount of ferrocyanide is formed in the absence of albumin. The ferrocyanide standard is therefore made up by adding ferrocyanide to a ferricyanide-Duponol PC solution which has been heated in exactly the same way as the ferricyanide-Duponol-albumin solution.

The experiments at pH 9.6 are carried out as follows. There are added to 1 cc. of 1 per cent dialyzed egg albumin 1 cc. of ferricyanide solution, 1 drop of 0.5 N NaOH, and 0.3 cc. of borate buffer. This solution is warmed to 37°C. and there is added to it 0.5 cc. of Duponol PC solution previously warmed to 37°C. Thus the denatured egg albumin is not exposed to air before it is exposed to ferricyanide. The experiment is completed in exactly the same manner as the experiments in neutral solution.

Formaldehyde.—To 1 cc. of 1 per cent dialyzed egg albumin there are added 0.5 cc. of 2 per cent Duponol PC, 2 drops of 0.5 N NaOH, and 6 drops of 37 per cent formaldehyde. After the solution has stood 5 minutes at room temperature there are added 0.2 cc. pH 6.8 phosphate, 1 drop of 1 N HCl, and 1 cc. of 0.05 M ferricyanide. The solution is allowed to stand 10 minutes at 37°C. and the ferric sulfate test is carried out as previously described. No Prussian blue is obtained.

Formaldehyde alone does not interfere with the Prussian blue reaction and does not reduce dilute ferricyanide at 37°C. Formaldehyde does reduce concentrated ferricyanide at 100°C.

Iodoacetamide.—Iodoacetamide is added to a neutral solution of egg albumin denatured by HCl or Duponol PC and the reducing power of the denatured egg albumin which has been treated with iodoacetamide is then tested with ferricyanide.

1 cc. of 1 N HCl is added to 4 cc. of 3 per cent dialyzed egg albumin. The test tube containing the solution is placed in 50°C. water for 2 minutes. The solution is cooled, neutralized with NaOH to green to brom thymol blue, and there are added 0.6 cc. pH 6.8 phosphate buffer, 2 cc. of 0.06 M iodoacetamide, and water to 12 cc. The solution is allowed to stand various times at 37°C. before samples are taken for the ferricyanide test. For the ferricyanide test there are added to 1 cc. of the albumin-iodoacetamide solution 3 drops of 1 M NaH_2PO_4, 0.3 cc. of 1 M pH 6.3 phosphate buffer, 0.5 cc. of 0.2 M ferricyanide, and 0.5 cc. of 10 per cent Duponol PC. After the solu-

tion has stood 10 minutes at 37°C. the ferric sulfate is added for the development of Prussian blue.

If the denatured albumin is treated for 5 minutes with iodoacetamide, about 0.0002 mM of ferrocyanide is formed. If the iodoacetamide reaction is carried out for 30 minutes, no ferrocyanide is formed.

As a control, the acid denatured egg albumin is allowed to stand 30 minutes in neutral solution in the absence of iodoacetamide. Then Duponol and ferricyanide are added and the normal 0.001 mM of ferrocyanide obtained. The control experiment shows that the reducing groups of denatured egg albumin are not appreciably oxidized by air in 30 minutes.

The experiment with egg albumin denatured by Duponol PC is carried out as follows. To 1 cc. of 4 per cent dialyzed egg albumin there are added 1 drop of 0.5 N NaOH, 0.3 cc. of pH 6.8 phosphate buffer, 1 cc. of 10 per cent Duponol PC, and 0.6 cc. of 0.06 M iodoacetamide and water to 4 cc. 1 cc. samples are removed after the solution has been allowed to stand for various times at 37°C. To the 1 cc. sample there are added 1 drop of NaH_2PO_4, 0.3 cc. of pH 6.3 phosphate, 0.5 cc. of 0.2 M ferricyanide, and 0.5 cc. of 5 per cent Duponol PC. The ferric sulfate test is carried out after the solution has stood 10 minutes at 37°C.

If the denatured egg albumin is treated with iodoacetamide for 30 minutes in neutral Duponol PC, about 0.0001 mM of ferrocyanide is still formed from the ferricyanide. If the iodoacetamide reaction is carried out for 180 minutes no Prussian blue is obtained. Whether Duponol PC has a small direct effect on the iodoacetamide reaction which slows it up, or changes the pH slightly, has not been investigated.

As a control, the denatured egg albumin is allowed to stand 180 minutes in neutral Duponol solution in the absence of iodoacetamide and then a mixed solution of ferricyanide and iodoacetamide is added either at pH 6.8 or at pH 6.3. Again 0.001 mM of ferrocyanide is obtained, as would be obtained in the absence of iodoacetamide. This control shows not only the stability of the SH group in the absence of iodoacetamide but also shows that iodoacetamide does not interfere with the ferricyanide reaction. At pH 6.3, 0.00095 mM ferrocyanide is formed even when the ferricyanide is added 1 minute after the iodoacetamide.

Table III shows the amounts of ferrocyanide formed when ferricyanide is added under various conditions to several amino acids and to several proteins which do not contain cysteine. The experiments are carried out in exactly the same way as the experiments with egg albumin.

SUMMARY

The following facts have been established experimentally.

1. In the presence of the synthetic detergent, Duponol PC, there is a definite reaction between *dilute* ferricyanide and denatured egg albumin. 0.001 mM of ferrocyanide is formed by the oxidation of 10 mg. of denatured egg albumin despite considerable variation in the time, temperature, and pH of the reaction and in the concentration of ferricyanide.

2. If the concentration of ferricyanide is sufficiently high, then the

reaction between ferricyanide and denatured egg albumin in Duponol solution is indefinite. More ferrocyanide is formed the longer the time of reaction, the higher the temperature, the more alkaline the solution, and the higher the concentration of ferricyanide.

3. Denatured egg albumin which has been treated with formaldehyde or iodoacetamide, both of which abolish the SH groups of cysteine, does not reduce dilute ferricyanide in Duponol PC solution.

4. Cysteine is the only amino acid which is known to have a definite reaction with ferricyanide or which is known to react with dilute ferricyanide at all. The cysteine-free proteins which have been tried do not reduce dilute ferricyanide in Duponol PC solution.

5. Concentrated ferricyanide oxidizes cystine, tyrosine, and tryptophane and proteins which contain these amino acids but not cysteine. The reactions are indefinite, more ferrocyanide being formed, the higher the temperature and the concentration of ferricyanide.

6. The amount of ferrocyanide formed from denatured egg albumin and a given amount of ferricyanide is less in the absence than in the presence of Duponol PC.

7. The amount of ferrocyanide formed when denatured egg albumin reacts with ferricyanide in the absence of Duponol PC depends on the temperature and ferricyanide concentration throughout the whole range of ferricyanide concentrations, even in the low range of ferricyanide concentrations in which ferricyanide does not react with amino acids other than cysteine.

The foregoing results have led to the following conclusions which, however, have not been definitely proven.

1. The definite reaction between denatured egg albumin in Duponol PC solution and dilute ferricyanide is a reaction with SH groups whereas the indefinite reactions with concentrated ferricyanide involve other groups.

2. The SH groups of denatured egg albumin in the absence of Duponol PC react with iodoacetamide and concentrated ferricyanide but they do not all react rapidly with dilute ferricyanide.

3. Duponol PC lowers the ferricyanide concentration at which the SH groups of denatured egg albumin react with ferricyanide. The SH groups of denatured egg albumin, however, are free and accessible even in the absence of Duponol PC.

REFERENCES

Anson, M. L., 1939*a*, *Science*, **90**, 256.
Anson, M. L., 1939*b*, *J. Gen. Physiol.*, **23**, 239.

Burk, N. F., 1937, *J. Biol. Chem.*, **120**, 63.
Folin, O., and Malmros, H., 1929, *J. Biol. Chem.*, **83**, 115.
Graff, S., Maculla, E., and Graff, A. M., 1937, *J. Biol. Chem.*, **121**, 81.
Greenstein, J., 1938, *J. Biol. Chem.*, **125**, 501.
Kassell, B., and Brand, E., 1938, *J. Biol. Chem.*, **125**, 145.
Kuhn, R., and Desnuelle, P., 1938, *Z. physiol. Chem.*, **251**, 14.
Mason, H. L., 1930, *J. Biol. Chem.*, **86**, 623.
Michaelis, L., and Schubert, M. P., 1934, *J. Biol. Chem.*, **106**, 331.
Mirsky, A. E., and Anson, M. L., 1935, *J. Gen. Physiol.*, **19**, 437.
Mirsky, A. E., and Anson, M. L., 1936, *J. Gen. Physiol.*, **19**, 451.
Neuberger, A., 1938, *Biochem. J.*, London, **32**, 1435.
Smythe, C. V., 1936, *J. Biol. Chem.*, **114**, 601.