LXXXI. THE REDUCING POWER OF CYSTEINE.

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(Received March 9th, 1928.)

THE physiologic significance of glutathione has been of the greatest interest ever since this compound was isolated by Hopkins [1921]. It has been shown to influence oxidative processes *in vitro*, and Dixon and Quastel [1923] and Dixon [1927] have published investigations concerning the oxidation-reduction potentials of glutathione and cysteine.

Kendall and Nord [1926] described some experiments concerning the reducing power of glutathione and cysteine and stated that both the —SH and —SS forms of these compounds are relatively stable. Cysteine and GSH cannot reduce indigo carmine and the —SS groupings cannot act as oxidising agents in a phosphate buffer of p_H 7.4. In the presence of indigo carmine, however, it was shown that the addition of hydrogen peroxide or sodium disulphide produces a third component in the solution which activates cysteine and GSH so that they can readily reduce indigo carmine and the —SS grouping can act as an oxidising agent; furthermore, the oxidation-reduction potential of the system is determined by the ratio of —SH to —SS if this third component is present in the solution. It was suggested that the third component is an oxygen or sulphur addition product of the —SH grouping.

Dixon and Tunnicliffe [1927] have recently criticised this work. They state that the published results do not warrant the conclusions and that the work was performed in an inaccurate manner with faulty technic and impure preparations. They characterised the suggested mechanism as novel and unnecessary for the explanation of the activity of the —SH and —SS groupings. Under the circumstances it appears necessary to reply with quantitative evidence to each of the many points raised by Dixon and Tunnicliffe.

OXYGEN IN THE "OXYGEN-FREE" NITROGEN.

Dixon and Tunnicliffe attribute the failure of Kendall and Nord to observe the reduction of indigo carmine by cysteine and GSH to the presence of oxygen in the nitrogen which was passed through the solutions. They estimate that the probable volume of gas passing through the solutions in 40 hours is 144 litres of nitrogen, and that the probable oxygen content of this is 15 cc., an amount sufficient to maintain the indigo carmine in the oxidised form.

They state that they have encountered difficulty in completely removing all traces of oxygen from nitrogen and assume that 0.01 % is a possible figure for the oxygen content of the nitrogen used by Kendall and Nord.

Before the investigation was started by Kendall and Nord an apparatus was constructed in such a manner that there was a large factor of safety in all details. For the purification of the nitrogen three Pyrex glass tubes, 2.5 cm. in diameter and 1 metre in length, were filled for a distance of approximately 60 cm. with fine copper-oxide wire. The section of the tube containing the copper was completely filled with the copper oxide so that there was no space between the oxide and the side of the Pyrex tube. The oxide was reduced with hydrogen; the three Pyrex tubes were placed side by side and each was wrapped with resistance wire and placed in a suitable asbestos-lined box so that a temperature between 300° and 400° could be constantly maintained. The diameter of the tubes was sufficient to permit a slow flow of gas through them and the subsequent reduction of copper oxide by passing hydrogen through the tubes in a direction opposite to that of the nitrogen showed that the copper was oxidised only in the first two tubes. Water was formed but rarely by reduction of copper oxide in the third tube. The ends of these Pyrex tubes were closed with a tapering glass seal to a 10 mm. glass tube bent downward making a U, and water forming in each of the copper-filled tubes would collect in this cooler U-tube giving an indication of the relative amount of copper oxide reduced by the hydrogen. Nitrogen, therefore, had to pass through approximately 180 cm. of metallic copper in a highly active form. The nitrogen passed through no rubber tubing in its course from the furnace to the solution. Copper tubing and glass with de Khotinsky's cement seals completely excluded all traces of oxygen.

Clark and Cohen [1923] have shown that this type of furnace is efficient for the removal of oxygen from nitrogen. We have many quantitative determinations proving the complete absence of oxygen from the nitrogen after passing through the furnace. A most severe criterion is the stability of 1 cc. of 0.004 *N* reduced indigo in 100 cc. of phosphate buffer, p_H 7.4. Such a solution will produce a reduction potential on the platinum electrode of approximately -0.28 v. Oxygen-free nitrogen may be passed through such a solution constantly for 8 hours without a significant change in the potential and without the separation of any indigo. Similar experiments have been performed with other solutions exceedingly sensitive to traces of oxygen. A platinum electrode which has been treated with cathodic polarisation in a solution of cysteine is highly sensitive to traces of oxygen. The small amount of oxygen contained in 1 cc. of distilled water, added to this solution will cause a marked decrease in the potential; however, the oxygen-free nitrogen may be passed through this solution for many hours without any decrease in the potential (see Fig. 1, p. 654).

ACTIVATION OF THE —SH GROUP WITH HYDROGEN PEROXIDE AND IRON.

Dixon observed the reduction of indigo carmine with cysteine in 1922. In a recent paper [1927] Dixon also states that cysteine is capable of reducing indigo carmine. In order to show that oxygen, hydrogen peroxide, or sodium disulphide is unnecessary for the activation of the —SH group, Dixon and Tunnicliffe prepared an apparatus consisting of three compartments; in one was placed a neutral cysteine solution, in the second a small amount of indigo carmine and in the third a strong solution of alkaline sodium hydrosulphite. Air was removed from the apparatus with a water pump and nitrogen was admitted. This operation was repeated twice and the solutions then remained in a vacuum for 24 hours. The indigo carmine and cysteine were mixed and it was observed that the dye was reduced within 6 minutes. Dixon and Tunnicliffe concluded that this observation renders unnecessary the assumption of any oxygen addition product.

In order to test our samples of cysteine and indigo carmine a similar apparatus was prepared and amounts equivalent to those which Dixon and Tunnicliffe used were placed in the two arms. The apparatus was evacuated, filled with nitrogen and this operation was repeated twice. It was then allowed to stand in the vacuum for some hours. The solutions were mixed, but no reduction of the indigo carmine occurred during several hours. The original experiments of Kendall and Nord were then repeated. Indigo carmine was added to an oxygen-free solution of cysteine at a p_H of 7.4 and it was found that the solutions were not reduced in the electrode chamber within a period of 24 hours.

This result is diametrically opposed to that of Dixon and Tunnicliffe and in order to determine whether our sample of indigo carmine was unique we secured 15 samples of this compound from several retail chemical supply houses. They were prepared by the following manufacturing firms: four samples from Grüber, six samples from the National Aniline Company, three samples from Coleman & Bell, one sample from Merck & Company, one sample from Eimer & Amend (manufacturer not stated).

A 0.02 *N* solution of each of these samples was prepared and added to cysteine in electrode chambers. Not one of the samples was reduced by the cysteine. We may therefore conclude that the original sample of indigo carmine was not unique. We then prepared several other samples of cysteine, according to the method of Gortner and Hoffman [1925]. 2 kg. of human hair was hydrolysed with 4000 cc. of 20 % hydrochloric acid until the biuret reaction was negative, usually about 130 hours. The solution was filtered, sodium acetate was added and the cystine which separated was dissolved in hydrochloric acid, treated with norite, and reprecipitated with sodium acetate. The purification was continued until the crystals were colourless. The cystine was reduced by heating 24 g. for 20 hours with tin moss added to 75 cc. of concentrated hydrochloric acid and 100 cc. of water. The tin was removed

by hydrogen sulphide in a volume of 1800 cc. The solution was filtered through norite and was then made to a volume such that 1 cc. contained 12 mg. of cysteine. The solution, therefore, was 0.1 *N* in respect to cysteine and it generally was equivalent to a 0.35 *N* solution of hydrochloric acid. The hydrochloric acid was neutralised by an equivalent amount of oxygen-free *N* sodium hydroxide just previous to using the cysteine solution.

By a new method which has been developed in this laboratory by V. B. Dowler, it was shown that this solution contained no cystine when freshly prepared. After standing even in the hydrochloric acid solution for several weeks a small amount of cystine was present. The iron was not removed from this solution by the method of Sakuma although iron was removed from some of the samples of cysteine. With six samples of cysteine prepared as outlined it was impossible to reduce even 2% of its molecular equivalent of indigo carmine.

Dixon and Tunnicliffe suggest that the main phenomenon observed by Kendall and Nord "is dependent on the presence of traces of iron present as impurities, perhaps as simple iron salts, perhaps as a cysteine-iron complex." It is difficult to interpret the suggestion that iron was the essential substance bringing about the reduction since no reduction was obtained in the absence of oxygen. However, the influence of iron on the reaction between cysteine and indigo carmine was investigated and it was found that when 1 cc. of 0.02 *N* ferrous sulphate or ferric chloride was added to a solution containing 6 cc. of 0.1 *N* cysteine and 3 cc. of 0.02 *N* indigo carmine in 100 cc. of phosphate buffer, p_H 7.4, there was no effect on the potential and the indigo carmine was not reduced.

That iron affects the reaction, however, may be readily shown and the experiment leaves little doubt concerning the reaction mechanism that is influenced by the iron. Indigo carmine, which is purified by recrystallisation from water, contains but minute traces of iron. Cysteine may be purified until it contains mere traces of iron, and when these two are mixed together no reduction of the indigo carmine occurs. If to such a solution hydrogen peroxide is added, or if air is admitted to the space above the solution, the indigo carmine is reduced but slowly. The cysteine and indigo carmine cannot be made to react with any appreciable velocity by the addition of hydrogen peroxide provided iron is removed as completely as possible. If traces of ferrous or ferric salts are added to a solution of cysteine in phosphate buffer, p_H 7.4, previous to the addition of hydrogen peroxide the indigo carmine is not reduced. If molecular oxygen or hydrogen peroxide is now added to the solution containing cysteine, indigo carmine and traces of iron, prompt reduction of the dye occurs. It is therefore evident that the function of iron is to decompose the hydrogen peroxide or activate molecular oxygen so that it can enter into the reaction between indigo carmine and cysteine.

This mechanism will be further discussed in connection with the influence of physical factors and the effect of iron expressed quantitatively is shown in Fig. 1 (p. 654). The sample of indigo carmine used by Kendall and Nord was

not free from iron; however, the presence of iron cannot explain the reduction of indigo carmine and the influence of iron in the reaction between oxygen and indigo carmine does not alter the conclusions concerning the necessity of activating the sulphhydryl group. Dixon and Tunnicliffe conclude this discussion of the reduction of indigo carmine by the statement: "We consider that these results dispose of Kendall and Nord's contention that cysteine and glutathione cannot reduce indigo carmine in the absence of oxygen."

The results which have been outlined are completely at variance with those of Dixon and Tunnicliffe. However, the barrier so sharply separating the two conflicting results is surmountable. Each of the 15 samples of indigo carmine can be reduced by each of the six preparations of cysteine if traces of oxygen, hydrogen peroxide, sodium disulphide or other activating agent is added to the solution. It would therefore appear that if the objective is the preparation of solutions containing cysteine which are stable in the presence of indigo carmine Kendall and Nord have had the good fortune to attain that objective and Dixon and Tunnicliffe's failure must be regarded as a negative result. Until they have shown that at least one solution of indigo carmine free from oxygen is stable in the presence of cysteine, the conclusion is justified that their solutions are of such a nature that activation of the —SH group is brought about by an unrecognised impurity. As will be shown, they have not excluded oxygen as the probable activating agent, and we have found that sodium disulphide, colloidal sulphur, and some organic compounds, such as adrenalone, can form a third component in the solution and function as activating compounds. These compounds as well as oxygen influence the reduction of indigo carmine by cysteine.

We can make no concrete suggestion at this time concerning the impurity activating the solutions used by Dixon and Tunnicliffe.

THE INFLUENCE OF PHYSICAL FACTORS. AGITATION.

In order to secure quantitative data concerning the effect of agitation, 6 cc. of 0.1 *N* cysteine were added to eight electrode cells containing 100 cc. of phosphate buffer, p_H 7.4, and sufficient sodium hydroxide to neutralise the hydrochloric acid in the cysteine solution. 1 cc. of 0.02 *N* ferrous sulphate was added to electrode cells 1, 2, 3 and 4; 3 cc. of 0.02 *N* indigo carmine was added to all the electrode cells. The potential in each cell was approximately —0.14 v. Five 2 cc. portions of 0.02 *N* hydrogen peroxide were added at intervals of about 20 minutes to electrode cells 3, 4, 7 and 8. During the addition of the peroxide the solution was agitated with a rapid stream of nitrogen passed through the solution. To electrode cells 1, 2, 5 and 6, 10 cc. of 0.02 *N* hydrogen peroxide were added at once and the agitation with nitrogen was stopped for 30 minutes. A distinct band of reduced indigo carmine formed in electrode cells 1 and 2 within a few minutes, but in cells 5 and 6 containing no ferrous sulphate the indigo carmine was reduced much more slowly. After the solutions in cells 1, 2, 5 and 6 were almost all reduced they were again agitated

with nitrogen and the velocity of oxidation of reduced indigo was then determined in all eight electrode cells. Since cells 1, 2, 3 and 4 contained iron, this experiment combines the influence of iron and that of agitation. The velocity of oxidation in cells 2, 4, 5 and 8 is shown in curves 2, 3, 4 and 5, respectively, in Fig. 1. It is assumed that the concentration of the third component is measured by the velocity of oxidation of the reduced indigo by the activated —SS grouping. That this is true will be shown presently. Since the concentration of the —SS grouping is the same in all cells, the difference in the velocity of oxidation must be due to the quantitative difference in the activation of the —SS grouping. It is apparent that iron has a great effect on the formation of the third component and that agitation does influence the reaction.

Fig. 1. The influence of iron and agitation on the formation of the third component.

Each of the solutions represented by curves 2, 3, 4 and 5 contained 6 cc. of 0.1 *N* cysteine in 100 cc. of phosphate buffer, p_H 7.4. Solutions 2 and 4 each contained 1 cc. of 0.02 *N* ferrous sulphate and 3 cc. of 0.02 *N* indigo carmine. 10 cc. of 0.02 *N* hydrogen peroxide were added in five 2 cc. portions to solution 3, while the solution was agitated with nitrogen. The same volume of peroxide was added to solution 2 without agitation. The difference in the velocity of oxidation of reduced indigo between solution 2 and solution 3 is due to the difference in the concentration of the third component. This difference is caused by agitation of the solution during the formation of the third component. Solutions 4 and 5 are similar to solutions 2 and 3, respectively, except that no ferrous sulphate was added to solutions 4 and 5. The only difference between solutions 4 and 5 is the factor of agitation, during formation of the third component. Solution 4 was not agitated. The difference between solutions 4 and 2, and 5 and 3, respectively, shows the influence of increased iron content. Solution 1 contained 1 cc. of 0.02 *N* reduced indigo only and the steady potential is good evidence that oxygen was not present in the nitrogen.

Since cysteine alone will reduce hydrogen peroxide without the formation of the third component, and since the peroxide must first react with the indigo carmine in order to form the third component, it is evident that two competing reactions are going on simultaneously in the solution. Any influence, therefore, which can increase the amount of peroxide reacting with the indigo carmine and prevent its interacting first with the —SH group will increase the concentration of the third component in the solution. Iron is one factor increasing the velocity of interaction between hydrogen peroxide and indigo carmine. If agitation decreases the amount of peroxide interacting with indigo carmine this would appear to be the probable mechanism by which

agitation inhibits the formation of the third component. Whether or not this is the correct explanation does not alter the fact that agitation does modify in a significant manner the formation of the third component in these solutions.

Not only does the formation of the essential third component depend on agitation but the concentration of the hydrogen peroxide added is also of importance; 2 cc. of 0.1 *N* hydrogen peroxide do not result in as high a concentration of the third component as 10 cc. of 0.02 *N* hydrogen peroxide; furthermore, Nord [1927] has shown that the activating compound is thermolabile and a solution which is capable of reducing indigo carmine when prepared as already outlined is found to have lost its power to reduce indigo carmine if the solution is heated to boiling for 5 minutes. The boiling must be carried out in the complete absence of oxygen. After cooling to the temperature of the thermostat such a solution cannot reduce more than traces of 0.02 *N* indigo carmine, but it can be reactivated by the subsequent addition of oxygen.

In this experiment the assumption was made that the velocity of oxidation of reduced indigo is proportional to the concentration of the third component in the solution. In order to show that this assumption is correct eight electrode cells were prepared with the same amounts, 3 cc. 0.1 *N* cysteine and 3 cc. 0.1 *N* cystine in 100 cc. phosphate buffer, p_H 7.4. To these solutions the following amounts of a solution containing the third component were added: cell 1, 0 cc., cell 2, 3 cc., cell 3, 6 cc., cell 4, 9 cc., cell 5, 12 cc., cell 6, 18 cc., cell 7, 24 cc. and cell 8, 30 cc. The preparation of the solutions is given on p. 663. Fig. 2 shows the velocity of oxidation of 1 cc. of 0.02 *N* reduced indigo in cells 1, 2, 3, 4, 5, 7 and 8. The curves are good evidence that the concentration of the third component is the determining factor in the velocity of oxidation of reduced indigo with cystine.

Dixon and Tunnicliffe confirmed the statement made by Kendall and Nord that indigo carmine is more rapidly reduced by cysteine if hydrogen peroxide is added to the solution; however, since they were able to reduce indigo carmine without the addition of oxygen or hydrogen peroxide the accelerating effect of hydrogen peroxide was considered by them as of little significance. They point out that the action of hydrogen peroxide may be either on the cysteine or on the indigo carmine. In order to determine which of these compounds was affected by the peroxide they prepared a solution of indigo carmine treated with dilute hydrogen peroxide. The excess peroxide was destroyed with manganese dioxide and it was then found that this solution, filtered free from the manganese dioxide, accelerated the reduction of indigo carmine. They state that this result shows conclusively that by the action of hydrogen peroxide on indigo carmine products are formed which have a marked catalytic effect on the reduction of indigo carmine by cysteine, and they say, "we consider that this fact offers the full explanation of the phenomenon observed by Kendall and Nord and renders unnecessary the assumption of the formation of the active oxygen addition product with cysteine."

We have repeated Dixon and Tunnicliffe's treatment of indigo carmine with hydrogen peroxide, removed the excess peroxide with manganese dioxide, and have confirmed their observation that this solution accelerates the reduction of indigo carmine. However, we have performed two other experiments with this solution and the result of this work emphasises the necessity for the assumption of something in the solution other than an oxidation product of indigo carmine.

Fig. 2. The influence of the concentration of the third component on the velocity of oxidation of reduced indigo by seven solutions each containing 100 cc. phosphate buffer, p_H 7.4, 3 cc. of 0.1 *N* cysteine and 3 cc. of 0.1 *N* cystine.

0, 3, 6, 9, 12, 24, 30 cc. of a solution containing the third component were added to the solutions 1, 2, 3, 4, 5, 6 and 7 respectively. The increased velocity of oxidation is due to activation of the —SS grouping by the third component. Although not shown in the figure the velocity of oxidation of reduced indigo by 30 cc. of the solution containing the third component alone was not greater than that shown in solution 3. The preparation of the solution of the third component is given on p. 663.

The first experiment was to allow the oxidation of 0.02 *N* indigo carmine with an equal volume of 3 % hydrogen peroxide and 1 cc. of 0.02 *N* ferrous sulphate to proceed until the solution was entirely bleached. The peroxide was then destroyed with manganese dioxide and 6 cc. of this solution were added to 100 cc. of phosphate buffer, p_H 7.4, containing 6 cc. of 0.1 *N* cysteine and 3 cc. of 0.02 *N* indigo carmine. The bleached indigo carmine solution did not permit the reduction of the indigo carmine by the cysteine. The experiment was repeated and the results are good evidence for the fact that the accelerating action of hydrogen peroxide and indigo carmine is not due to oxidised indigo carmine alone.

The second experiment which bears on this point was performed as follows. 0.02 *N* indigo carmine was bleached with an equal volume of 3 % hydrogen peroxide and 1 cc. of 0.02 *N* ferrous sulphate until one-half of the indigo carmine had been destroyed. The extent of bleaching of the indigo carmine

was determined by diluting the solution and matching in a colorimeter, using the original solution suitably diluted as a standard. The remaining hydrogen peroxide was then destroyed with manganese dioxide and it was shown that the filtrate was able to catalyse the reduction of indigo carmine by cysteine. A portion of this solution was boiled after the excess of hydrogen peroxide had been removed and before it was deoxygenated. After boiling, which had produced no change in the colour of the solution, it was found that cysteine could no longer reduce the indigo carmine. This result is in agreement with the experiment already described in which the third component was destroyed by boiling a solution containing cysteine, cystine and the third component, and is evidence that some thermolabile intermediate product is the essential agent which activates the —SH group. This will be discussed somewhat more in detail when the effect of sodium disulphide is described, and in the last section of this paper.

We have also confirmed Dixon and Tunnicliffe's observation that iron greatly accelerates the action of hydrogen peroxide on indigo carmine. It was interesting to note that the indigo carmine which we used was so free from iron that 0.02 *N* hydrogen peroxide had no bleaching power and when the 0.02 *N* indigo carmine solution was mixed with an equal volume of 3 % hydrogen peroxide it reacted so slowly that it could stand 20 hours without destruction of more than 50 % of the indigo carmine.

THE RÔLE OF SODIUM DISULPHIDE IN THE FORMATION OF THE ADDITION COMPOUND.

It is difficult to reply to Dixon and Tunnicliffe's comments on the action of sodium disulphide since their reference to the paper of Kendall and Nord is so contrary to the statements made in that paper. Through an oversight in the preparation of the manuscript the method used in the preparation of sodium disulphide was omitted, but, since there is only one way by which this compound can be prepared readily, this omission was not misleading. The sodium disulphide was prepared by the addition of sulphur to a solution of sodium sulphide. The method used, in brief, was as follows. A solution containing 40 % of sodium hydroxide was saturated with hydrogen sulphide. The solution was then diluted to such an extent that when it was cooled crystals of sodium sulphide separated in large flat plates on the bottom of the container. If the solution was too concentrated the crystals separate in a solid mass. The solution was diluted so that but a small percentage of the sodium sulphide separated. The crystals were separated from the solution by decantation and were washed with a small amount of distilled water which was immediately decanted.

10 g. of crystals were then weighed roughly on a balance and dissolved in 100 cc. of water. This solution was then titrated for its sodium sulphide content with 0.1 *N* iodine. It was then made to 0.7 *N*. To 50 cc. of the 0.7 *N* sodium sulphide solution the equivalent amount of flowers of sulphur was

added. The sulphur dissolved readily, forming a yellow solution; this was diluted with distilled water until it was 0.01 *N*.

The statement of Dixon and Tunncliffe that sodium disulphide is a powerful reducing agent requires more definite description of the conditions used in determining its reducing action. A solution of 0.01 *N* sodium disulphide added to 100 cc. of buffer, p_H 7.4, immediately hydrolyses with the precipitation of colloidal sulphur and liberation of hydrogen sulphide. Although hydrogen sulphide is a reducing agent capable of reducing indigo carmine, sulphur is an oxidising agent capable of oxidising reduced indigo carmine. If oxygen-free nitrogen is passed through the solution the hydrogen sulphide is readily removed and the colloidal sulphur is left in suspension.

If indigo carmine is present in the solution when the sodium disulphide is added, slight, and only slight, reducing action is evident. 1 cc. of 0.02 *N* indigo carmine in 100 cc. of phosphate buffer, p_H 7.4, is not reduced by five times its equivalent of sodium disulphide; on the other hand, the precipitated sulphur is an oxidising agent when a sufficiently sensitive criterion is used, namely, reduced indigo or reduced indigo carmine. The precipitated sulphur will oxidise its full equivalent of reduced indigo, and after each addition of reduced indigo the potential will return to the value of approximately -0.14 v. It was stated by Kendall and Nord [1926, p. 320] that "feeble ability to reduce indigo carmine and approach the same equilibrium point" was also shown, that is -0.14 v.

If, however, the reducing power of sodium disulphide is tested in distilled water instead of phosphate buffer of p_H 7.4, the higher hydroxyl concentration prevents hydrolysis and colloidal sulphur does not separate. The solution ceases to have oxidising power and under these conditions it is, as Dixon and Tunncliffe say, "a most powerful reducing agent."

The two statements of Dixon and Tunncliffe, first, that sodium disulphide is a powerful reducing agent in the condition in which it was used by Kendall and Nord, and second, that no control experiments with sodium disulphide alone were made, are both incorrect. Fig. 8 in the paper by Kendall and Nord is devoted entirely to the oxidising action of sodium disulphide, and the effect on indigo carmine is clearly stated. However, even though sodium disulphide was a powerful reducing agent at p_H 7.4 and could reduce its full equivalent of indigo carmine, still the explanation of the reduction of indigo carmine given by Dixon and Tunncliffe is impossible since the amount of sodium disulphide added was in each case less than the amount of indigo carmine used.

Since sodium disulphide cannot reduce indigo carmine at p_H 7.4, the reduction of this dye with cysteine and sodium disulphide cannot be explained except on the basis of activation of the $-SH$ group.

It has now been found that colloidal sulphur prepared by acidifying sodium disulphide with its equivalent of sulphuric acid and completely removing the hydrogen sulphide is also capable of activating the sulphydryl group so that it can reduce indigo carmine. Iron does not increase or prevent the activating action of sodium disulphide or colloidal sulphur.

Colloidal sulphur is not a sufficiently powerful oxidising agent to oxidise indigo carmine in the same way as does hydrogen peroxide. The effect cannot be solely on indigo carmine and must involve the sulphhydryl group. It is for this reason that it seemed most probable that the interaction of hydrogen peroxide and indigo carmine does not involve, primarily and only, the indigo carmine; its effect on the sulphhydryl group is due to a definite change in the intensity relations within the sulphhydryl group itself, the result of which is to enable it to enter into chemical reactions and to exert reducing action which cannot be demonstrated in the absence of suitable activation.

CONTROL OF HYDROGEN ION CONCENTRATION.

The criticism of the control of hydrogen ion concentration does not appear to be well taken. The acidity or alkalinity of all solutions prior to their introduction into the electrode chambers was determined by titration with 0.1 *N* acid or alkali, using phenol red as indicator. If 6 cc. of the sodium salt of cystine were added to the solution, 6 cc. of deoxygenated 0.1 *N* sulphuric acid were subsequently added and, although it was not stated in the paper, the hydrogen ion concentration of the solution was determined by colorimetric comparison after this procedure and it was found that the p_H of the solution was not affected. If the hydrochloride of cysteine was used, an equivalent amount of deoxygenated sodium hydroxide was added to the solution in some cases; in other cases a definite amount of hydrochloric acid was left unneutralised, but, if so, the same known amount of acid was present in all eight electrode cells used in each experiment. Fig. 3 in the paper of Kendall and Nord shows the influence of adding 6 cc. of 0.1 *N* sulphuric acid to the solution and such a change in the p_H does not alter the characteristic poisoning action of the solution and in no way alters the conclusions drawn. The charts and statements included in the paper of Kendall and Nord were selected from a large mass of data and were chosen to show definite influences on the —SH and —SS groupings. Many controls and variations in the technic were carried out but could not be included. Dixon and Tunnicliffe give no experimental results to show that in any way the procedure followed by Kendall and Nord resulted in lack of control of the hydrogen ion concentration, and the procedure followed was stated by Kendall and Nord in detail sufficient to permit duplication of the results.

THE USE OF INDIGO REDUCED WITH GLUCOSE AND ALKALI.

Dixon and Tunnicliffe state that the method of preparation of solutions of reduced indigo which Kendall and Nord used in experiments on the reversibility of —SH and —SS systems seems also to call for some notice, and finally that "it is obvious that such a solution containing one of the most powerful reducing agents, glucose, in the presence of alkali together with the unknown decomposition product of glucose will give potentiometric readings of little significance."

Before the solution of reduced indigo was used for this work careful controls were carried out concerning the quantitative value of its reduction potentials. The action of alkali and glucose causes the complete solution of all the indigo added to the solution and, provided it is a preparation of synthetic indigo of high purity, the concentration of the reduced indigo in solution is equivalent to the quantity used. 1 cc. of 0.02 *N* reduced indigo solution, prepared as previously described, by the use of glucose and sodium hydroxide, added to 100 cc. of oxygen-free phosphate buffer, p_H 7.4, will produce a reduction potential equivalent to approximately -0.26 to -0.28 v. If to this 1 cc. of 0.02 *N* solution of an oxidising dye is added, for example, indigo carmine, methylene blue or dibromophenolindophenol, the potential drops immediately to a point depending on the E_0 value of the oxidising dye. As soon as all the reduced indigo is oxidised with indigo carmine the potential decreases at least 0.15 v., and with the other oxidising dyes the drop in potential after complete oxidation of the reduced indigo may be as great as 0.3 v. Instead of a solution containing "one of the most powerful reducing agents" as stated by Dixon and Tunnicliffe, actual observations show that the maximal reduction potential of such a solution, except for the reduced indigo, is not more than -0.12 v. This may be readily shown by preparing such a solution using all the constituents except the indigo; in other words, when 4.5 g. of glucose is boiled with 45 cc. of *N* sodium hydroxide and 250 cc. of water, through which oxygen-free hydrogen is passed, and the boiling is continued for 5 minutes after the last addition of the glucose to the alkali, the maximal reduction intensity of this solution is not greater than -0.12 v.

Other evidence showing conclusively that the reduced indigo solution, prepared with glucose, contains no substance which can affect the quantitative values in potentiometric titrations, as it was used, is afforded by titration of the reduced indigo with indigo carmine, the reduced indigo being added to 10 cc. of indigo carmine in the electrode cell. It is apparent that, by adding the reduced indigo to the dye, all substances in the solution which can be oxidised at an intensity equal to that of the dye will cause a reduction of indigo carmine. If, then, the reverse procedure is carried out, the indigo carmine being added to 10 cc. of the reduced indigo, the high reducing power of the reduced indigo will remove all oxidising power of the indigo carmine before the indigo carmine can oxidise reducing substances in the solution of less reducing intensity than reduced indigo. The addition of indigo carmine is stopped when the potential of -0.16 v. indicates complete oxidation of the reduced indigo. When such a technic is followed it is found that the indicated strength of the reduced indigo is identical by both methods. If anything other than reduced indigo were present which could be oxidised with indigo carmine this relationship could not hold. When the same experiment is tried with dibromophenolindophenol it is found that less reduced indigo solution is required when it is added to 10 cc. of the dibromo-dye. This is evidence that, if the maximal oxidation potential of the dye is greater

than approximately -0.12 v., substances in the reduced indigo solution formed by the action of alkali on the glucose will reduce some of the dye. This fact does not affect the accuracy of the method as used for solutions of cystine.

Since the maximal oxidising intensity of the $-SS$ grouping in the presence of reduced indigo carmine is less than the maximal reducing intensity of the glucose solution by at least 0.05 v., it is obvious that there is no reducing compound in the glucose solution which can be oxidised by the $-SS$ grouping; on the other hand, the entire amount of reduced indigo can be oxidised by the $-SS$ grouping at a level of intensity above the maximal reduction intensity of the other constituents of the glucose solution.

10 cc. of 0.02 *N* reduced indigo in 100 cc. of phosphate buffer, p_H 7.4, containing 0.1 *N* acid, just sufficient to bring the p_H of the reduced indigo to 7.4, can be accurately titrated with 0.02 *N* dibromophenolindophenol, 0.02 *N* methylene blue or 0.02 *N* indigo carmine. The volume of oxidising dye required to decrease the potential to a value -0.16 v. (indicating the complete oxidation of the reduced indigo) is the same with these three dyes, and affords an accurate assay of the indigo in the solution.

Whether or not the formation of products from the oxidation of glucose affects the reaction is another matter; such an effect has been shown on oxidation of adrenaline and related compounds, but after using solutions of reduced indigo for 3 years we have observed no such effect on the $-SH$ grouping and the solution has justified its use in many ways. Control blanks on the stability have shown that it is stable in the phosphate buffer solution for many hours, and the results which we have obtained on the oxidising power of the $-SS$ group and the results published by Kendall and Nord cannot be lightly dismissed.

Another point which should be emphasised is the fact that all of these solutions were compared on a relative basis; if a series of eight electrode cells containing various solutions were titrated with reduced indigo it was the differences in the velocity of oxidation which indicated the activity of the $-SS$ grouping. The potential following the addition of 1 cc. of 0.02 *N* reduced indigo to 100 cc. of phosphate buffer, p_H 7.4, is shown in curve 1, Fig. 1. Oxygen-free nitrogen was passed through the solution for 4 hours without significant change in the potential. 1 cc. of 0.02 *N* indigo carmine oxidised the reduced indigo and produced an immediate drop in potential to -0.15 v. Similar evidence that the nitrogen used by Kendall and Nord was free from oxygen is given in Fig. 9 of their paper. 3 cc. of 0.02 *N* reduced indigo was not oxidised by passing nitrogen through the solution for 6 hours.

All of the conclusions of Kendall and Nord were based on the relative velocity of the change in potential and were not concerned with the total volume of reduced indigo which was oxidised. For a study of such reactions it is imperative that the reducing agent used be a substance of such a nature that the oxidised form produced by the reaction will not accumulate in the

solution and thereby influence the equilibrium point of the solution. Reduced indigo is such a substance and the criticisms of Dixon and Tunnicliffe, although applicable in the presence of a stronger oxidising agent than the —SS grouping, do not affect the value or interpretation of the results obtained with this reagent.

It is difficult to understand Dixon and Tunnicliffe's criticism of the alkalinity of the reduced indigo solution. Instead of stating that neutralisation of reduced indigo was unnecessary, Kendall and Nord included in the Figs. 1, 2, 3 and 4 the amount and time when the acid was added to compensate for the alkalinity of the reduced indigo added; the letter "J" was used to denote the addition of acid to the solution. The statement of Dixon and Tunnicliffe, that the alkalinity cannot be determined by an indicator, has no bearing on the procedure followed. They state that the addition of 40 % caustic soda failed to change the colour of phenol red added to the solution after removal of the indigo. This statement is true but is without significance, as the addition of only 2 to 3 cc. of 0.1 *N* sulphuric acid to 5 cc. of the reduced indigo solution after removal of the indigo will change the indicator in a satisfactory manner and indicate the amount of sodium hydroxide present. They estimated that in a sample of reduced indigo which they prepared the p_H was approximately 12; this hydroxyl ion concentration is less than that of a 0.1 *N* solution of alkali; therefore, for each 5 cc. of reduced indigo, less than 5 cc. of oxygen-free 0.1 *N* sulphuric acid would be required to make the resultant p_H in the solution the same as before the addition of the reduced indigo solution.

From the first use of reduced indigo it has been the practice in this laboratory to determine the alkalinity of each preparation. As ordinarily prepared, 5 cc. of such a solution requires between 2.0 and 3.0 cc. of 0.1 *N* sulphuric acid to bring it to neutrality, using phenol red as indicator; this amount of acid has been added after the addition of each 5 cc. of reduced indigo. No error can result from this procedure which can in any way alter the conclusions concerning the oxidising power of the —SS grouping. A quantitative measure of the effect of the slight alkalinity resulting from the addition of 5 cc. of reduced indigo solution is shown in Fig. 1. At 7 hours 37 minutes 2.8 cc. of 0.1 *N* sulphuric acid were added to the solutions in cells 2 and 3. The reduction potential of the solution had been increased 5 millivolts by the 5 cc. of reduced indigo above the potential indicated at 4 hours 35 minutes. Addition of the sulphuric acid again brought the potential to the precise value shown 3 hours previously, although the equilibrium potential had been displaced four times during this interval by an amount equal to more than twenty times the effect of the alkali in the reduced indigo. Kendall and Nord submitted evidence to show that in a solution containing an activating agent the —SS grouping is capable of acting as an oxidising agent. The paper of Dixon and Tunnicliffe does not show that the —SS grouping cannot act in this manner.

THE INFLUENCE OF THE —SH/—SS RATIO.

Dixon and Tunnicliffe state that examination of the experimental evidence of Kendall and Nord fails to establish the conclusion that it is the ratio of the —SH to the —SS grouping which determines the potential in solutions containing the activating component. The potentiometric evidence for this statement will be given in another paper; however, there is other evidence which has a direct bearing on this point. If 25 cc. of 0.1 *N* cysteine hydrochloride and 2 cc. of 0.02 *N* ferrous sulphate are placed in 162 cc. of phosphate buffer, p_H 7.4 (with sufficient sodium hydroxide to neutralise the hydrochloric acid), in a 500 cc. flask containing an inlet tube, a delivery tube, and an outlet on the side of the neck, and the solution is then deoxygenated, 12.5 cc. of oxygen-free 0.02 *N* indigo carmine can be added through the side tube on the neck without reduction of the indigo carmine. If to such a solution 37.5 cc. of 0.02 *N* hydrogen peroxide are added without agitation the indigo carmine is promptly reduced. Before and after the introduction of the hydrogen peroxide, oxygen-free nitrogen is passed through the flask and out through the tube on the neck. The nitrogen is purified in a special small copper furnace and the gas passes through nothing except copper tubing or glass after it has been freed from oxygen. The third component is produced in the solution and the indigo carmine is completely reduced to a canary yellow colour. This solution is transferred to a movable burette, similar to one described by Clark and Cohen, and 30 cc. are placed in each of eight electrode cells. The colour is uniform. If to six of this series of cells 1 cc. of 0.1 *N* cysteine and 1, 2, 3, 4, 5, 6 cc. of 0.1 *N* cystine (as the sodium salt) and the corresponding amount of oxygen-free 0.1 *N* sulphuric acid are added, a series of solutions will be prepared in which the ratio of the —SH to the —SS grouping varies within wide limits. The amount of cysteine and the p_H are the same in all. Such a series of solutions shows a perfect gradation in the potential of the equilibrium point; moreover, it shows a corresponding change in the colour of the indigo carmine. The added cystine changes the —SH/—SS ratio and this in turn changes the ratio of the reductant/oxidant forms of indigo carmine.

A comparison of the colours of the solution is good evidence that the oxidation-reduction intensity in the solution is determined by the —SH/—SS ratio. If to one of the remaining two cells, 3 cc. of 0.1 *N* cysteine are added, there will be an increase in the reduction potential and a still further intensifying of the canary yellow colour resulting from the reduction of the indigo carmine. If 1 cc. of 0.1 *N* cystine but no cysteine is added to the remaining cell, the potential is decreased more than in cell 1. The colour corresponds. Not only is the colour of the solution determined by the ratio —SH/—SS, but the velocity of oxidation of the reduced indigo is tremendously accelerated in those solutions containing the higher concentrations of the —SS grouping. Further evidence concerning this will be given in a paper on the potentiometric measurements.

ACTIVATION OF —SH AND —SS GROUPINGS.

The work described in the preceding part of this paper furnishes conclusive evidence that the velocity of oxidation of cysteine is influenced by other constituents in the solution which have the power to activate the sulphydryl group. In order further to study the effect of oxygen on this reaction an apparatus similar to the one described by Dixon and Tunncliffe was constructed, the form of which is shown in Fig. 3. No rubber stoppers were used but the two openings were closed with ground glass tubing to which glass stopcocks were fused. The apparatus consisted of four compartments which made it possible to add activating substances, such as hydrogen peroxide, sodium disulphide or colloidal sulphur on one side only. One half always served as a check for the other. By the use of a high vacuum pump capable of producing a vacuum equivalent to a fraction of a millimetre of

Fig. 3.

mercury, not only were oxygen and nitrogen removed, but the water vapour formed by the rapid evaporation of about one-third of the solution carried out from the apparatus all traces of oxygen which could be removed by this means. Nitrogen was admitted and the apparatus was again evacuated. This was repeated three times, and the experiment was then carried out in a vacuum. The amounts of cysteine, indigo carmine and activating solutions used are given in Table I. In one side of the apparatus, compartments A , A' , were placed the buffer, cysteine and one-half of the water; on the other side, compartments B , B' , were placed the ferrous sulphate, indigo carmine, one-half of the water and the activating agent. When the apparatus was tipped A' mixed with B' , A with B . The volume given in the table shows the volume of each constituent after mixing. The sodium hydroxide is given in terms of normal solution but where the volume was less than 0.5 cc., 0.1 N solution was used. All solutions which did not reduce in the time given in the chart were immediately activated by the addition of hydrogen peroxide and a small amount

of iron. In each case the indigo carmine was reduced following this treatment. The last 13 experiments were performed with a solution of the same composition in each of the four compartments. The solutions which were present at the same time in the apparatus are indicated in the Table by *A* and *B*.

Table I. *The time required for reduction of indigo carmine by activated cysteine.*

	0.1 N cysteine, cc.	0.1 M ferrous sulphate, cc.	N sodium hydroxide, cc.	Phosphate buffer, pH 7.6, cc.	Water, cc.	0.2 N indigo carmine, cc.	0.2 N hydrogen peroxide, cc.	0.2 N colloidal sulphur, cc.	0.01 N sodium disulphide, cc.	0.01 M adre- naline, cc.	Time required for reduction
<i>A</i>	0.5	0.1	0.17	3.0	10.0	0.5	—	—	—	—	Not in 18 hrs.
<i>B</i>	0.5	0.1	0.17	3.0	10.0	0.5	1.0	—	—	—	20 mins.
<i>A</i>	1.0	0.1	0.35	3.0	10.0	0.5	1.0	—	—	—	20 mins.
<i>B</i>	1.5	0.1	0.52	3.0	10.0	0.5	1.0	—	—	—	15 mins.
<i>A</i>	2.0	0.1	0.70	3.0	10.0	0.5	1.0	—	—	—	10 mins.
<i>B</i>	2.5	0.1	0.875	3.0	10.0	0.5	1.0	—	—	—	10 mins.
<i>A</i>	1.5	0.1	0.52	3.0	10.0	0.5	—	—	1.0	—	10 mins.
<i>B</i>	0.0	0.1	—	3.0	10.0	0.5	—	—	1.0	—	Not reduced
<i>A</i>	1.5	0.1	0.52	3.0	10.0	0.5	—	2.0	—	—	5 mins.
<i>B</i>	0.0	0.1	—	3.0	10.0	0.5	—	2.0	—	—	Not reduced
<i>A</i>	1.5	—	0.52	6.0	5.0	0.5	—	—	1.0	—	5 mins.
<i>B</i>	1.5	—	0.52	6.0	5.0	0.5	—	—	—	—	Not in 60 mins.
<i>A</i>	2.5	—	0.87	3.0	10.0	0.5	—	—	—	2.27	75 mins.
<i>B</i>	2.5	—	0.87	3.0	10.0	0.5	—	—	—	0.45	90 mins.
	0.0	—	—	3.0	10.0	0.5	—	—	—	—	Not in 105 mins.
	2.5	0.1	0.87	3.0	10.0	0.5	—	—	—	—	16 hrs.
<i>A</i>	1.5	—	0.52	6.0	5.0	0.5	—	—	—	—	8 hrs.
<i>B</i>	2.5	0.1	0.87	3.0	10.0	0.5	—	—	—	—	2½ hrs.
	2.5	0.1	0.87	3.0	14.0*	0.5	1.0	—	—	—	7 mins.
<i>A</i>	2.5	0.5	0.83	3.0	5.0	1.0	0.5	—	—	—	15 mins.
<i>B</i>	2.5	—	0.83	6.0	10.0	1.0	0.5	—	—	—	2 hrs.
<i>A</i>	2.5	0.1	0.87	3.0	14.0*	0.5	1.0	—	—	—	5 mins.
<i>B</i>	2.5	0.1	0.87	3.0	14.0*	0.5	—	—	—	—	Not in 5 hrs.
<i>A</i>	2.5	0.1	0.87	3.0	10.0	0.5	—	—	—	—	Not in 4 hrs.
<i>B</i>	2.5	0.1	0.87	3.0	10.0	0.5	—	—	—	—	80 mins.
	2.5	—	0.83	6.0	10.0	1.5	—	—	—	—	Not in 10 hrs.
											20 mins.
<i>A</i>	2.5	0.1	0.87	3.0	10.0	0.5	—	—	—	—	Not in 12 hrs.
<i>B</i>	2.5	0.1	0.87	3.0	10.0	0.5	—	—	—	—	9 hrs.
<i>A</i>	1.5	0.1	0.52	3.0	10.0	0.5	—	—	—	—	9 hrs.
<i>B</i>	1.5	0.1	0.52	3.0	10.0	0.5	—	—	—	—	7½ hrs.
	2.5	—	0.87	3.0	10.0	0.5	—	—	—	—	1 hr.
	5.0	—	1.66	3.0	20.0	1.0	—	—	—	—	All four compart- ments in 45 mins.
	1.3	—	1.1	1.6	5.5	0.3	—	—	—	—	5 hrs. 25 mins.
	1.3	—	1.1	1.6	5.5	0.3	—	—	—	—	6 hrs. 10 mins.
	1.3	—	1.1	1.6	5.5	0.3	—	—	—	—	6 hrs. 10 mins.
	1.3	—	1.1	1.6	5.5	0.3	—	—	—	—	6 hrs. 25 mins.
	1.5	—	0.5	1.8	6.0	0.3	—	—	—	—	2 hrs. 30 mins.
	1.5	—	0.5	1.8	6.0	0.3	—	—	—	—	5 hrs.
	1.5	—	0.5	1.8	6.0	0.3	—	—	—	—	8 hrs. 45 mins.
	1.5	—	0.5	1.8	6.0	0.3	—	—	—	—	9 hrs. 10 mins.
	2.1	—	0.7	2.5	4.2	0.4	—	—	—	—	2 hrs. 30 mins.
	2.1	—	0.7	2.5	4.2	0.4	—	—	—	—	2 hrs. 50 mins.
	2.1	—	0.7	2.5	4.2	0.4	—	—	—	—	3 hrs. 15 mins.
	2.1	—	0.7	2.5	4.2	0.4	—	—	—	—	3 hrs. 20 mins.

* 4 cc. of water evaporated while in a vacuum.

The difference in the velocity of reduction was striking and was conclusive evidence that the activity of the —SH group was tremendously affected by the presence of the third component formed in the solution. However, in no

case did the indigo carmine in the unactivated solution remain in the oxidised form for more than a few hours.

The original experiments in which it was shown that cysteine cannot reduce indigo carmine were again carried out in electrode vessels through which oxygen-free nitrogen was continuously passed. Although the phosphate buffer, cysteine and indigo carmine were present in the same amounts as were used in the vacuum apparatus, the indigo carmine remained in the oxidised form for 72 hours. Furthermore, when this solution was prepared in the movable burette, which was used to add solutions to the electrode chambers, it was found that the indigo carmine remained in the oxidised form for more than 48 hours. A solution containing the indigo carmine and a second solution containing the cysteine, after being thoroughly deoxygenated, were mixed in an electrode cell which had been deoxygenated; the solution was then passed back into the movable burette and further deoxygenated. After this treatment the indigo carmine remained in the oxidised form although no nitrogen was passed through the solution. When air or hydrogen peroxide was admitted to these solutions, which had remained for from 48 to 72 hours unchanged, the indigo carmine was completely reduced within 5 minutes.

After confirmation of the observation that indigo carmine is not reduced by cysteine in the absence of oxygen when the solutions are held in electrode cells or in the movable burette, further experiments were made to determine the time required to reduce the indigo carmine by cysteine in the vacuum apparatus. The significant observation was then made that the time required for the reduction of indigo carmine varied greatly for the same solution in the four different compartments; the difference was sometimes as great as 6 hours. Furthermore, the indigo carmine in small drops of the solution which adhered to the cross tube of the apparatus was always promptly reduced. These observations were explained by the assumption that, even though the apparatus had been held in a high vacuum for several hours, sufficient oxygen remained adsorbed on the surface of the glass to bring about prompt reduction in a small volume of the solution and that the varying velocity of reduction in the four compartments was due to the varying amounts of oxygen adsorbed on the glass.

One further series of experiments was then made in order to remove all oxygen from the surface of the apparatus. A solution of glucose, to which sodium hydroxide and indigo carmine were added, was placed in the apparatus, completely filling all parts, including a short section of rubber tubing attached to the inlet tube. The alkaline glucose reduced the indigo carmine and, after this powerful reducing agent had remained in contact with the glass for a few hours, the apparatus was thoroughly washed with distilled water which had been previously deoxygenated with oxygen-free nitrogen. The water was added to the apparatus through the movable burette. After several washings all traces of alkaline glucose were removed and, through the use of the same

movable burette, it was possible to add the mixture of indigo carmine and cysteine without exposure to oxygen. At no time was any part of the apparatus except the rubber inlet tube exposed to air during the deoxygenation and subsequent washing out of the reducing solution. The solution containing cysteine-indigo carmine was equally divided in the four arms of the apparatus which was then evacuated. It was observed that the time required for the reduction of the indigo carmine was greatly extended, but still complete reduction occurred after about 9 hours. It then became evident that oxygen cannot be sufficiently excluded by this type of experiment to establish the fact that cysteine cannot reduce indigo carmine in the complete absence of oxygen.

During this work it was noted that if a trace of vaseline remained on the surface of the glass it was impossible to remove the oxygen from the glass and vaseline. On one occasion a small particle of vaseline broke off from the glass stopcock and floated on the surface of the solution. This was the focus from which the activation of the solution could be followed and prompt reduction of the indigo carmine occurred in an ever-widening circle surrounding this minute source of oxygen. These results show that when an apparatus of this type is used the conclusion that cysteine can reduce indigo carmine in the complete absence of oxygen is not justified. The activation of a solution containing cysteine and indigo carmine is such a delicate reaction that it will serve as a test for the minutest traces of oxygen.

It doubtless is possible to prepare an apparatus similar to the one described in which indigo carmine will remain in the oxidised form for 72 hours, but the extreme precautions which would have to be used would probably require fused glass connections throughout. These obstacles have prevented our carrying out this experiment and since the crucial experiment is furnished by the use of the moveable burette as well as the electrode cell, we regard these results as sufficiently conclusive to justify the statement that in the complete absence of oxygen and other activating agents cysteine cannot reduce indigo carmine.

From these results we conclude that hydrogen does not dissociate from cysteine with the formation of cystine except in the presence of compounds which can suitably interact with the sulphydryl group. Interaction with the sulphydryl grouping may be brought about by agents other than indigo carmine and oxygen, such as colloidal sulphur and possibly substances which may occur as impurities in cysteine or indigo carmine.

SUMMARY.

Cysteine cannot reduce indigo carmine and cystine cannot oxidise reduced indigo carmine or reduced indigo in the absence of a third component which functions as an activating agent. This activating agent is produced through the action of oxygen or sodium disulphide on indigo carmine.

Iron cannot activate the —SH or —SS grouping, but iron markedly influences the velocity of reaction between oxygen and indigo carmine. This results in an increased concentration of the activating agent. Agitation of the solution and other physical factors influence the formation of the activating agent. It is destroyed by heat. Iron neither accelerates nor prevents the action of sodium disulphide on indigo carmine.

The chemical nature of the activating agent appears to be an unstable oxygen or sulphur addition product of indigo carmine, and its essential action involves the activation of the sulphur atom, which permits the sulphydryl and disulphide groupings to manifest their latent reducing or oxidising power. In the presence of a constant concentration of the activating agent the velocity of oxidation and reduction is determined by the ratio C.SH/C.SS.C groupings.

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