REVERSIBLE OXIDATION-REDUCTION SYSTEMS OF CYSTEINE-CYSTINE AND REDUCED AND OXIDIZED GLUTATHIONE.

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Investigation of the oxidation-reduction potentials of 2-oxy-2,3-dihydroindole-3-propionic acid established the fact that the compound in both its reduced and oxidized forms has little effect on the platinum electrode (9). It has the ability, however, to react with dibromoindophenol, with hydrogen dioxide, molecular oxygen, and many other oxidizing agents. Interaction with these compounds produces within the solution variations in the reducing potential. When dibromoindophenol is used, the dye is completely reduced and a reducing potential of +0.1 volts can be produced. The oxidation of this lactam was shown to be of a simple stoichiometric nature, and the velocity curve indicated that an addition product was formed between the lactam and the oxidizing compound, probably through the amine group. It was suggested that this intermediate product is unstable, tending to break down with the removal of two atoms of hydrogen from the lactam.

The reduced and oxidized forms of 2-oxydihydroindole-3-propionic acid are not in equilibrium with each other, and they do not form a reversible oxidation-reduction system. No compound has been found which can bring the two forms into equilibrium.

In 1923, Dixon and Quastel published an investigation of the oxidation-reduction potentials of cysteine and cystine and of reduced and oxidized glutathione (4). They suggested that these

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compounds belong to a new system of substances, only one form of which (the reduced) affects the platinum electrode. They stated that the effect of adding oxidized glutathione to reduced glutathione is to increase the reducing potentials, but they do not give any interpretation of this fact. In their conclusions they state that the reducing potentials of cysteine and of glutathione are independent of the presence or absence of their oxidized forms and that the variation of the reducing potentials with concentration of RSH and pH is given by the relation

$$\pi = \pi_0 + \frac{RT}{F} \log (\text{H}^+) - \frac{RT}{F} \log c,$$

where $\pi$ is the observed potential, $\pi_0$ is the normal reduction potential and is a constant, and $c$ is the concentration of RSH. They give determinations in their paper which appear to show that the actual concentration of cysteine determines the reducing potential and that the addition of cystine to the cysteine has no effect.

When it was shown that the oxidized form of 2-oxy-2,3-dihydroindole-3-propionic acid did not affect the platinum electrode, it seemed probable that this lactam resembled, at least in some respects, cysteine and cystine. In the hope of finding other relationships between cystine and the indole derivative under investigation, experiments were made similar to those of Dixon and Quastel with cysteine and cystine.

It was first shown that cystine not only did not affect the platinum electrode, but it was remarkably stable toward reducing agents. When reduced indigo was added to the electrode chamber containing cystine, a high reducing potential was produced. It was not changed by the cystine and no poising action could be demonstrated.

In order to determine the reducing power of cysteine, similar experiments were performed in which oxidizing dyes were used in place of reduced indigo and it was shown that 2,6-dibromoindophenol reacts with cysteine in a definite stoichiometric relation. The dibromoindophenol is reduced and the cysteine is oxidized in equivalent amounts. Other dyes were substituted for dibromoindophenol and among these indigo carmine was used. It was then found that although cysteine could rapidly reduce the derivatives of indophenol, it was unable to reduce indigo carmine.
Addition of cystine to the cysteine had no effect on the ability of cysteine to react with indigo carmine.

In the course of these experiments, which were repeated several times and which were frequently continued for more than 24 hours, it was noticed that in two of the eight electrode chambers in the thermostat the indigo carmine was reduced after the solutions had stood overnight, without oxygen-free nitrogen passing through them. Apparently some activating agent was present in these two solutions which enabled the cysteine to reduce the indigo carmine. The experiment was repeated, the solutions being allowed to stand overnight without oxygen-free nitrogen passing through them. On the following morning, as before, two or three of the solutions were colorless and all the remaining solutions were deep blue.

On the surface of the solutions which were reduced, there was a thin layer of the solution of blue unreduced indigo carmine. This suggested that the reduction of the indigo carmine might be explained by the presence of minute amounts of oxygen, this being the most probable agent which could have entered the electrode chamber. This observation was confirmed directly as follows: The solutions were prepared and allowed to stand for several hours in order to show that they would not reduce indigo carmine. The reduction of the dye was then brought about by the intentional addition of 10 cc. of air, displacing an equivalent amount of oxygen-free nitrogen in the space above the solution in the electrode chamber. When this was done the reducing potential began to increase, and after about 2 hours the solution turned greenish-yellow in a band about 2 cm. below the surface of the solution. This band continued to increase in depth so that the reduction of the dye could be followed to the bottom of the electrode chamber. The indigo carmine just below the surface of the solution was the last to be reduced, but in all the electrode chambers practically complete reduction of the dye occurred after the oxygen, from 10 cc. of air, had been absorbed into the solution. After this experiment had been repeated many times, the air was added in other ways. When the air was mixed with the nitrogen and bubbled through the solution, little or no reduction of the indigo carmine occurred. The explanation for the retarding effect of stirring during the addition of oxygen was not secured for many weeks. This is discussed further on.
During the early experiments, the activating influence of oxygen on a solution containing cysteine and indigo carmine was shown by preparing the solutions in an empirical way. The buffer was thoroughly deoxygenated with oxygen-free nitrogen and the cysteine solution in the form of its hydrochloride was also de-oxygenated; they were then mixed and a solution of oxygen-free indigo carmine in varying amounts was added to the solution. The nitrogen above the solution was then displaced with air, and the electrode chambers were permitted to stand without agitation until reduction of the indigo carmine occurred.

All of our results indicated that molecular oxygen was indispensable for the activation of cysteine, enabling it to reduce indigo carmine; and, since Thurlow has shown that oxygen reacts with reduced glutathione, forming hydrogen dioxide, the next step in the investigation was to add hydrogen dioxide, instead of molecular oxygen, to the solutions of cysteine (1). Hydrogen dioxide 0.01 N was therefore added to solutions of cysteine, and after some time indigo carmine was added. No reduction of the indigo carmine occurred. The indigo carmine was then added to the cysteine solution before the hydrogen dioxide. With the conditions thus produced, the indigo carmine was reduced almost immediately, providing sufficient hydrogen dioxide had been used. If small amounts of hydrogen dioxide were used the reduction occurred more slowly, and if the solutions were agitated the reduction was retarded in proportion to the agitation. The effect of agitation, preventing molecular oxygen from activating the solution described above, was therefore confirmed by the use of hydrogen dioxide instead of molecular oxygen.

These results indicate the existence of an essential intermediate compound which is formed by the action of hydrogen dioxide on cysteine. This compound cannot be an activated form of cysteine alone and it cannot be cystine, since mixtures of cysteine and cystine are quite without effect upon indigo carmine. The active compound, therefore, must be an oxygen addition product which is formed by the interaction of hydrogen dioxide and cysteine. For many months we were unable to prepare this compound in the absence of indigo carmine. The reason for this is discussed further on. The oxygen addition compound is capable of acting as a catalytic agent and in its presence cysteine rapidly reduces indigo carmine.
When the solutions are prepared under proper conditions large amounts of indigo carmine are rapidly and completely reduced. Under slightly altered conditions, however, cysteine may fail completely to reduce indigo carmine. The failure of hydrogen dioxide to form the essential intermediate compound under any but suitable conditions, even in the presence of indigo carmine, is well illustrated by the slow addition of the hydrogen dioxide, with agitation, to a solution of cysteine. Under these conditions the potential of the solution before the addition of the dioxide may be approximately $-0.138$ volts. Addition of from 1 to 2 cc. of 0.01 N hydrogen dioxide solution causes a drop in the potential to approximately $-0.04$ volts, which then slowly rises to $-0.136$ volts. If more dioxide is added, the drop in the potential is produced, followed by a slow rise to $-0.136$ volts, which appears to be the limiting value under these conditions. This may be repeated many times with the same result. The value of $-0.136$ volts must be the maximal reducing potential of cysteine in the presence of not more than 10 per cent of its gram equivalent of indigo carmine.

*Equilibrium between Cysteine and Cystine.*

When hydrogen dioxide is added to varying amounts of cysteine or when a constant amount of cysteine is treated with varying amounts of hydrogen dioxide in such a manner that the oxygen addition product is formed, a series of solutions is obtained which shows in striking manner that the resulting reducing potential is determined by the ratio of the concentrations of cysteine and cystine. Although cysteine in the presence of small amounts of indigo carmine cannot produce a reducing potential higher than $-0.136$ volts and although cystine has apparently but little effect on the platinum electrode, both cysteine and cystine are brought into equilibrium if the oxygen addition product is present in the solution. This compound apparently acts as an essential link between the oxidized and reduced forms of the SH grouping.

12 cc. of 0.1 N cysteine and 1.4 cc. of 0.1 N indigo carmine in each of four electrode chambers were treated with 1, 2, 4, and 6 cc. of

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\(^1\) The potential is also dependent on the pH of the solution. For details see Experimental.
0.01 N hydrogen dioxide, respectively. Four more electrode chambers, containing 11, 10, 9, and 8 cc. of 0.1 N cysteine, respectively, and 1.4 cc. of 0.1 N indigo carmine, were all treated with 6 cc. of 0.1 N hydrogen dioxide. In this way a series of solutions was obtained in which the ratio of cysteine to cystine varied within wide limits. It was found that the potential of the solution containing 11 cc. of cysteine and 1 cc. of cystine was much higher than the reducing potential of the solution containing 2 cc. of cysteine and 6 cc. of cystine, these quantities being approximately the amounts formed by the hydrogen dioxide. The reducing potentials of the solutions between these two limits corresponded to the ratios of the cysteine to cystine present. It is therefore evident that the ratio of cysteine to cystine establishes the equilibrium point in these solutions.

If a further addition of indigo carmine is made to the solution containing 2 cc. of cysteine and 6 cc. of cystine, it produces but a slight drop in the reducing potential of the solution which slowly returns to the original value. However, if indigo carmine is added to the solution containing 11 cc. of cysteine and 1 cc. of cystine, there is a marked drop in the potential followed by a prompt return to the high value present before the addition of indigo carmine. After the solution has come again to equilibrium, the addition of indigo carmine may be repeated without appreciable change in the value of the reducing potential, until sufficient indigo carmine is added to cause a significant alteration in the ratio of the cysteine to cystine. As this ratio becomes smaller, the reducing potential of the solution approaches −0.136 volts.

Reversible Reduction-Oxidation System of Cysteine-Cystine.

After it had been shown that cysteine cannot reduce indigo carmine except in the presence of an oxygen addition product which acts as a catalyst, experiments were made to determine whether this same compound would act as an agent permitting the reduction of cystine by suitable reducing agents. Nowhere in the literature have we been able to find any reference to the reduction of cystine by organic substances not of animal origin at a temperature of 30° and a pH of 7.4 (7, 12). It has been shown that SH groups and hydrogen sulfide will reduce cystine but no system which
is reversible has been described (5). It was therefore with great
interest that a solution of reduced indigo was added, in an elec-
trode chamber entirely free from oxygen, to a solution containing
cysteine, cystine, and the oxygen addition compound. The
reducing potential of the solution before the addition of the
reduced indigo was $-0.160$ volts. The addition of 1 cc. of $0.02 \text{ N}$
reduced indigo raised the potential to $-0.244$ volts. After 1 or 2
minutes, during which time the potential reached a definite peak,
it began to diminish and after from 10 to 15 minutes the potential
was approximately the same as it was before the addition of the
reduced indigo. The change in potential was closely paralleled
by the separation of indigo. This could be repeated any number
of times provided sufficient cystine remained in the solution.

When a solution of reduced indigo is added to a solution of in-
digo carmine there is no delay in the oxidation of the reduced
indigo. Deviations from the original oxidizing potential are small
until after sufficient reduced indigo has been added to influence
appreciably the ratio of reduced to oxidized indigo carmine.
However, if a solution of reduced indigo is added to a solution
containing cysteine, cystine, and the oxygen addition product of
cysteine, there is a prompt increase in the reducing potential
depending on the amount of reduced indigo added. The equi-
librium point is not reached instantly as it is with reduced indigo
and indigo carmine, but there is a slow drift in the potential toward
a maximum; the time required to reach this maximum depends
on the ratio of cysteine to cystine. A solution containing a large
proportion of cysteine may require 4 or 5 hours to reach the max-
imal reading, whereas with a small amount of cysteine and a large
amount of cystine, the maximal reading may be reached within 2
minutes. Following the maximal value there is a drop in the
reducing potential. The velocity of this reaction also depends
on the ratio of cysteine to cystine and the concentration of the
oxygen addition product. If the solution contains a large amount
of the oxygen addition product the reduced indigo will all be
oxidized within 5 minutes. If, however, the concentration of the
oxygen addition product is low, several hours may be required
for the indigo to be completely oxidized. This is in striking con-
trast to the usual course of chemical reactions; for example, the
neutralization of acids and alkalis, or the reduction of indigo
carmine by reduced indigo (Figs. 1 and 2).
Fig. 1. Reversible oxidation-reduction in solutions of cysteine and cystine in the presence of the oxygen addition product.
Fig. 2. Reversible oxidation-reduction in solutions of cysteine and cystine in the presence of the oxygen addition product.
Glutathione

Source of Oxidizing Power.

When cysteine, in the presence of the oxygen addition product, reduces indigo carmine there can be no doubt that the source of the reducing action is the sulphydryl group. After it had been shown that reduced indigo could be oxidized by a solution containing cysteine, cystine, and the oxygen addition product, it became necessary to show that the source of energy for the oxidation of the reduced indigo was the SS grouping. This was readily done by determining the condition of the substances in the solution. The source of oxidation of the reduced indigo cannot be the hydrogen dioxide because the gram equivalents of reduced indigo that can be oxidized are many times greater than the gram equivalents of hydrogen dioxide added. Furthermore, it is impossible to regard hydrogen dioxide as the source of oxidation as it would rapidly react with cysteine and be completely reduced. The high reducing potential of the solution previous to the addition of reduced indigo is quantitative evidence that no trace of hydrogen dioxide was present. The source of oxidation cannot be indigo carmine because the color of the solution shows that the indigo carmine is completely reduced. Moreover, the reducing potential of the solution proves that indigo carmine is incapable of acting as an oxidizing agent. The only remaining source for the oxidizing power is the SS grouping. Reduced indigo can be added many times to a solution containing a small amount of cysteine and a large amount of cystine without material alteration in the velocity of oxidation or in the final equilibrium value of the solution, but the addition of reduced indigo to a solution containing a large amount of cysteine and a small amount of cystine results in a slow oxidation of the indigo and an appreciable alteration in the equilibrium point of the solution.

The results with indigo carmine established the fact that the energy of the SH grouping can be made available for reduction. The oxidation of reduced indigo demonstrated that the energy of the SS grouping can be made available for oxidation, and it was then easily shown that the same solution containing cysteine, cystine, and the oxygen addition product will function either as a reducing or an oxidizing agent, depending solely on the addition to the solution of substances which change the potentials a little.
above or below that of the equilibrium point of the solution of cysteine-cystine. Since the reducing potential of the solution of cysteine-cystine is determined quantitatively, after the formation of the oxygen addition compound, by the ratio of cysteine to cystine, it can be changed only temporarily by the addition of oxidizing or reducing agents. If indigo carmine is added to such a solution the reducing potential drops, but this is soon followed by an increase to the value present before the addition of indigo carmine. If then reduced indigo is added to the same solution there is an increase in the reducing potential followed by a decrease until it reaches the value present before the addition of the reduced indigo.

These results show that a truly reversible system is present and that the equilibrium value is reached in either direction with surprising promptness and with uncanny accuracy. The constancy of the equilibrium point in these solutions which are of a simple nature, free from protein and other complex substances, is not equaled by any other systems known to the authors. Solutions of similar constancy of equilibrium points are found only associated with biologic processes; for example the fixed pH of the blood and the normal hemoglobin or blood sugar value.

Reversible System of Reduced and Oxidized Glutathione.

After the existence of a reversible system of cysteine and cystine in the presence of the oxygen addition product had been established, experiments were made with reduced and oxidized glutathione. The results obtained were similar to those obtained with cysteine and cystine. Solutions having a fixed equilibrium point were produced, which would oxidize reduced indigo and reduce indigo carmine in a manner similar to solutions of cysteine and cystine (Figs. 3 and 4). There was no appreciable difference in the absolute value of the equilibrium points. The most oxidizing solutions approached the value of 0.140 volts which is approximately the value obtained with cysteine and cystine; however, there was one striking difference in the solutions of glutathione which appears to be significant. Oxygen can be removed from solutions of cysteine and cystine by passing oxygen-free nitrogen through the solution for from 40 to 80 minutes. When a solution of glutathione, prepared in this manner, is added to indigo car-
mine, the indigo carmine is rapidly reduced. This could be explained in one of two ways: either reduced glutathione can of itself and without the aid of the oxygen addition product reduce indigo carmine, or else it is difficult to remove the last traces of oxygen which are necessary and sufficient to enable the reduced glutathione to react with the indigo carmine. The latter explana-
tion is the correct one. If the solution of glutathione is deoxygenated for from 15 to 20 hours with oxygen-free nitrogen, and the indigo carmine contained in a buffered solution of pH 7.4 is similarly deoxygenated, the addition of the glutathione to the

Fig. 4. Reversible oxidation-reduction in solutions of reduced and oxidized glutathione in the presence of the oxygen addition product.

indigo carmine solution is followed by a decrease in reducing power and the indigo carmine remains oxidized for many hours. The addition of ferric chloride to such a solution causes a decrease in the reducing potential and in fact oxidizes the indigo carmine, which has been slightly reduced by the glutathione. This is
evidence that iron is not an essential in the formation of the oxygen addition product (11, 14, 15) (Fig. 5).

When hydrogen dioxide is added to the solution containing completely deoxygenated indigo carmine and glutathione, the glutathione is activated in a short time and the color of the solution changes in distinct layers, showing the complete reduction of the indigo carmine in definite portions of the solution. After standing a sufficient length of time the oxygen addition product appears to diffuse through the entire solution resulting in complete reduction of the indigo carmine. Complete deoxygenation of the glutathione appears to cause the solution to become more sluggish in its interaction with indigo carmine and hydrogen dioxide and the activities of oxygen-free solutions cannot be compared with those of freshly prepared solutions containing traces of oxygen.

The ease with which cysteine solutions can be deoxygenated emphasizes the great advantage of working with cysteine during the investigation of the factors determining its activity. A "yes" or "no" answer is easily obtained with cysteine and cystine. The blue solution of the indigo carmine remains blue for long periods of time or it promptly changes to pale yellow. With glutathione this is not so, unless it is completely deoxygenated. In the presence of minute traces of oxygen, glutathione will reduce indigo carmine, and the speed of the reduction is the only indication of a change in the activity of the solution. It is impossible, without taking extreme precautions, to obtain a "yes" or "no" answer.

Nature and Chemical Properties of the Functioning Form of Cysteine.

Certain properties of the oxygen addition product and the reducing and oxidizing activities of cysteine and cystine have been definitely established, which assist in formulating a working hypothesis to explain the activity of the three compounds. At no time have we been able to produce a system in which cysteine, cystine, and the oxygen addition product are in equilibrium, with a lower reducing potential than $-0.136$ volts. This figure, then, must have some quantitative bearing on the reaction. Within the limits from $-0.136$ to $-0.300$ volts the compound appears to be stable for many hours. Below the reducing potential of $-0.136$ volts it is unstable and apparently breaks down into cystine and water. Although hydrogen dioxide is essential for the formation
Fig. 5. Reduced glutathione cannot completely reduce indigo carmine in the absence of traces of oxygen, hydrogen dioxider, or sodium disulfide.
of this compound, the oxygen addition product is stable and can function only in a solution which has such a high reducing potential that hydrogen dioxide itself cannot exist.

If cysteine is present alone in a buffered solution, the reducing potential is found to vary from time to time and there is a constant drift toward a high reducing potential. If, however, completely deoxygenated indigo carmine is added and then a few drops of 0.01 N hydrogen dioxide are added to the solution, the reducing potential drops to a value very near -0.136 volts. The solution maintains this value without appreciable drift for many hours. From these experiments it may be concluded that in a solution buffered to pH 7.4, -0.136 volts is the reducing potential which can be produced by cysteine in the absence of the oxygen addition product. The corresponding figure for glutathione is approximately 0.150 volts. A solution with this reducing potential cannot completely reduce indigo carmine.

The stability of the CSS grouping is made manifest in striking manner by its inability to oxidize reduced indigo. Cysteine and cystine alone, therefore, do not form a reversible oxidation-reduction system. It can readily be shown, however, that the oxygen addition product will act as an agent permitting both reduction and oxidation, and its properties can be summarized in the statement that it behaves toward oxidizing and reducing substances and affects the platinum electrode in a manner entirely similar to other compounds which form reversible oxidation-reduction systems. This can occur only with a compound which can exist in a reduced and oxidized form.

In order to visualize more clearly the chemical properties of the oxygen addition product of cysteine, two possible structures of the molecule were considered, depending on whether oxygen or hydrogen dioxide combines with cysteine (8).

![Chemical structures](http://www.jbc.org/Downloadedfrom)
If formula I is correct, but a single product can result from its oxidation. By the loss of two atoms of hydrogen two molecules will combine:

\[
\begin{align*}
2 \text{C-S} & \rightarrow \text{C-S} - \text{O} \\
\end{align*}
\]

As will be pointed out later, however, a compound with this structure could not possess the properties which the oxygen addition product can be shown to have. A compound with formula II would be a substance which could exist in both reduced and oxidized forms. In the presence of mild oxidizing agents the four atoms of hydrogen could be removed and the molecule would become

\[
\begin{align*}
\text{C-S} & - \text{O} \\
\end{align*}
\]

This would involve the loss of two negative valence bonds in the atom of sulfur. The molecule existing in its oxidized form, however, could readily be reduced back to its tetrahydro derivative in the presence of hydrogen donators.

The known limits for the activity of the compound in regard to the oxidation and reduction potentials furnish a quantitative interpretation for this action. That the oxygen addition product of cysteine will act catalytically, permitting the reduction of indigo carmine, is shown by the fact that the addition of hydrogen dioxide will bring about the reduction of many times the equivalent amount of indigo carmine. The amount of indigo carmine that
can be reduced is related to the amount of cysteine in solution. However, the velocity of reduction of the indigo carmine is determined by the concentration of the oxygen addition product. The only explanation of the reduction of indigo carmine, therefore, is that the cysteine acts through the oxygen addition product which makes available the hydrogen of the sulfydryl group of cysteine. Therefore, one of the chemical properties of the oxygen addition product which must be accepted is that it will interact with cysteine and thereby sensitize the hydrogen of the sulfydryl group to a state of reactivity sufficient to reduce indigo carmine. If two molecules of cysteine combine with the oxidized form of the oxygen addition product, the molecule becomes

$$\text{C} - \text{S} \quad \text{C} - \text{S} - \text{S} - \text{C}$$

$$\text{O} \quad \text{O}$$

$$\text{O} \quad \text{O}$$

$$\text{C} - \text{S} \quad \text{C} - \text{S} - \text{C}$$

This is a derivative of the tetrahydro form of the oxygen addition product in which two of the hydrogen atoms are replaced by two positive groups. It is this reaction which would be impossible if the oxygen addition product possessed formula I.

When the reverse reaction is considered, that is, the oxidation of reduced indigo by means of cystine, still more striking evidence is at hand strengthening the hypothesis that it possesses the structure of formula II. Reduced indigo produces in a solution a reducing potential which at a pH 7.4 is approximately $-0.280$ volts. It can readily be shown that at pH 7.4 cystine is stable in the presence of reduced indigo unless the oxygen addition product is present, in which case the indigo is rapidly oxidized by means of the cystine. The function of the oxygen addition product, therefore, cannot be to produce within the solution simply a high reducing potential, because this is accomplished through reduced indigo. Cystine will oxidize reduced indigo at a reducing potential 100 millivolts less than the potential given to a solution by means
of reduced indigo alone. For these reasons it is necessary to assign to the oxygen addition product a unique power for interacting with cystine. This explanation is easily given provided the compound possesses the structure of formula II.

In a solution with a reducing potential in the neighborhood of \(-0.136\) volts, the oxygen addition product will exist largely in its oxidized form. If reduced indigo is added to the solution, the reducing potential immediately increases. The change in reducing potential produces a change in the valence of the sulfur atoms in the oxygen addition product. Each atom of sulfur tends to go to a higher negative valence but the group which combines with the sulfur is dependent on the concentration of cystine. The partially hydrogenated molecule resulting from interaction between reduced indigo and the oxygen addition product may react with cystine.

\[
\begin{align*}
\text{C-S} & \quad \text{H} \\
\text{O} & \quad \text{O} \\
\text{+ 2H} & \quad \rightarrow \\
\text{O} & \quad \text{O} \\
\text{C-S} & \quad \text{H}
\end{align*}
\]

In the solution under consideration the reducing potential is low and the dihydrodisulfydryl radical derivative of the oxygen addition product is unstable, readily breaking down as cysteine and again forming the oxidized form of the oxygen addition product.

If, however, this same reaction is pictured occurring in a solution with a reducing potential near \(-0.260\) volts, cysteine is present in a higher concentration than is cystine; in this condition the partially hydrogenated oxygen addition product would not react with cystine but could take up a second atom of hydrogen and form the tetrahydro derivative. The formation of the tetrahydro derivative is indicated by the fact that the solution develops a high reducing potential after the addition of the reduced indigo which is very slowly oxidized. However, the addition of indigo
carmine to such a solution is followed by a rapid reduction of the indigo carmine.

These reactions show that the latent oxidizing and reducing energy of the solution is made available through the oxygen addition product. Although cysteine and cystine alone cannot form a reversible oxidation-reduction system, the oxygen addition product in its oxidized and reduced forms does constitute such a system. Cysteine and cystine in solution under its influence act simply as reducing and oxidizing agents, and thus establish the equilibrium point. Under no conditions, however, can cysteine and cystine alone function as the agents which allow the system to be reversed.

The system, therefore, acts somewhat like buffers for the regulation of the concentration of hydrogen ions. However, there are striking differences indicating that the equilibrium is more fixed and is established by a different mechanism than in simple buffered solutions regulating the interactions of acids and bases. There is no buffered solution described, consisting of three components, which works in such a manner that to change from the first to the third component necessitates interaction with the second; but this appears to be the simplest possible mechanism that can explain the results obtained with cysteine, cystine, and the oxygen addition product.

Any compound which can act as a hydrogen donator added to the solution produces a high reducing potential, but before the added substance can react with the cystine present, it is necessary first to have its available hydrogen add to the \( \text{CSOOSC} \) grouping. When the oxygen addition product formed by the action of hydrogen dioxide on cysteine is recognized as a substance which can form a reversible oxidation-reduction system, the significance of the value \(-0.136\) volts, which is the lower limit at which this system can exist, becomes more apparent. It is obvious that, if cysteine is one of the reaction products of the oxidation of a hydrogen donator by cystine, the reducing potential of the solution can never be lower than the minimal value which is established by cysteine itself. At a pH of 7.4 this value is close to \(-0.136\) volts.

Evidence that cystine is the source of the oxidizing power converting the hydrogen donator, reduced indigo, into indigo, and
that the other product of this reaction is cysteine, is furnished by the fact that the value \(-0.136\) volts is the equilibrium point approached by a solution containing cystine in large amount, cysteine in small amount, and the oxygen addition product, after the reducing potential has been raised to a high figure by the addition of reduced indigo.

\textit{Reducing Potentials of Solutions of Cysteine.}

When cysteine or a mixture of cysteine and cystine is dissolved in a solution buffered to pH 7.4 and oxygen is excluded from the solution, the potential slowly increases. Dixon and Quastel first pointed out this change in potential and suggested the use of gold electrodes in order to overcome the technical difficulties in working with this substance (4). The potential drift in solutions of cysteine has been observed many times during this investigation, but until the properties of the oxygen addition product of cysteine had been established no explanation could be given. It now seems probable that the changing potential is caused by a change in the sulfydryl group of cysteine (5).

If two molecules of cysteine react with each other, a compound will result having the structure

\[
\begin{align*}
\text{H} & \\
\text{C - S - H} & \\
\text{C - S} & 
\end{align*}
\]

This formula resembles the structure assigned to the oxygen addition product of cysteine and it would probably have reducing properties which are much more marked than those of cysteine itself. The addition of a small amount of indigo carmine would react with this addition product of two molecules of cysteine and immediately eliminate the high reducing potential of the solution. Whether more of this addition product is then formed appears to depend on the substances in the solution. In the presence of small amounts of indigo carmine, the high reducing potential is not again developed. When hydrogen dioxide, dibromoindophenol, 1-naphthol-2-sulfonate-indo-2,6-dichlorophenol, or methylene blue, are added to solutions of cysteine the potential decreases im-
mediately, but as soon as the dioxide or dye has been reduced the former high potential is manifested.

**Formation of a Sulfur Addition Product with Cysteine.**

During the investigation of the reversible system of cysteine-cystine and the oxygen addition product, it was observed on more than one occasion that after the solutions had stood for 48 hours hydrogen sulfide was given off. It was simultaneously noted that the solution manifested increased activity in the oxidation of reduced indigo. Under no condition could hydrogen sulfide be regarded as the source of the increased oxidizing power of the solution, but the observation suggested the investigation of other sulfur compounds in the presence of cysteine and cystine. If hydrogen dioxide can combine with cysteine and form an oxygen addition product, hydrogen disulfide should be capable of forming an analogous sulfur compound.

In order to determine this, solutions of cysteine and cystine were prepared and to them 0.01 N sodium disulfide was added in amounts of 5 and 10 cc. Indigo carmine was then added but no reduction of the dye occurred. This experiment was repeated, but the indigo carmine was first added to the cysteine and cystine solutions before the addition of the sodium disulfide in the same amounts as before. Prompt reduction of the indigo carmine resulted. Further investigation showed that sodium disulfide reacts with cysteine forming an addition product similar in chemical properties to the oxygen addition product of cysteine. That cysteine reacts with sodium disulfide in the same manner as it does with hydrogen dioxide, is indicated by the fact that it is essential to have indigo carmine present in the solution when sodium disulfide is added.

If 0.01 N sodium disulfide is added to water buffered to pH 7.4 with phosphate, sulfur is precipitated and forms a milky suspension. In this finely divided form it remains in suspension for a long time. When cysteine was present in the solution, however, no turbidity was noticed, and it was then shown that if sodium disulfide is added to the buffer solution and the sulfur is precipitated, addition of cystine to the solution promptly brings about complete solution of the precipitated sulfur, resulting in a clear, colorless solution. This is further evidence of the combination
Fig. 6. Reversible oxidation-reduction in solutions of cysteine and cystine in the presence of the sulfur addition product.
Fig. 7. Reversible oxidation-reduction in solutions of reduced and oxidized glutathione in the presence of the sulfur addition product.
of sulfur with cystine, although of course it does not explain the chemical nature of the groups formed by the addition of sulfur.

The velocity of reduction of indigo carmine and oxidation of reduced indigo by cysteine-cystine and by GSH, GSS, which had been activated with sodium disulfide, is of the same order as the velocity of these reactions after activation with hydrogen dioxide. Whether selenium and tellurium would act in the same manner has not been determined (Figs. 6 and 7).

![Graphs showing oxidation-reduction potentials](image)

**Fig. 8. Reduced indigo is oxidized by sodium disulfide.**

**Change in the Valence of Sulfur in Reversible Oxidation-Reduction Systems.**

It has been shown that cysteine and cystine can be brought into equilibrium with each other by the presence of an oxygen or sulfur addition product, and the chemical properties of the addition products indicate that they can exist in oxidized and reduced forms, which are sensitive to reducing and oxidizing substances in the solution. This can be interpreted only by a change in the valence of the sulfur atom. Since the ultimate source of oxidation
Glutathione

and reduction in this system resides in the sulfur, still simpler forms of sulfur compounds should show similar oxidizing and reducing power. That this is true may be demonstrated with a dilute solution of sodium disulfide alone. A 0.01 N solution of sodium disulfide added to a solution buffered to pH 7.4 in an electrode chamber free from oxygen will oxidize reduced indigo, precipitating indigo from solution and will establish an equilibrium point in the scale of oxidation-reduction potentials, which is similar to the equilibrium point of solutions of cysteine and cystine (Fig. 8). Feeble ability to reduce indigo carmine and approach the same equilibrium point can also be shown. All of the oxidizing-reducing activity of the substances discussed in this paper may be directly related to a change in the valence of the atom of sulfur in the compounds. Under certain conditions the sulfur is capable of existing in such a form that it tends to change its valence and develop two negative valence bonds which are either maintained or lost, depending on the presence of other substances in the solution. The reason why cysteine and cystine do not of themselves reduce indigo carmine or oxidize reduced indigo, is because the sulfur in these molecules cannot change its valence, except under what may be called extreme treatment, as with tin and mineral acid. When hydrogen dioxide or sodium disulfide have been added to cysteine, the sulfur atom can respond to changes in oxidizing-reducing potentials in the solution, and the mechanism of the response consists in the utilization of the valence bonds which manifest their activity under the influence of the groups attached to the sulfur atom.

Function of Indigo Carmine in the Production of the Oxygen or Sulfur Addition Product.

A summary of the results shows that in no case has the oxygen addition product been produced either from cysteine or reduced glutathione in the absence of indigo carmine or some other hydrogen acceptor. Indigo carmine is as essential with sodium disulfide as it is with hydrogen dioxide. Our interpretation of this experimental result is as follows: When hydrogen dioxide or sodium disulfide reacts with cysteine or reduced glutathione, the immediate result of the action is the production of the tetrahydro derivative of the oxygen or sulfur addition product and this sub-
stance, unless it can be oxidized, immediately decomposes forming water or hydrogen sulfide and the S-S grouping. The function of indigo carmine is to act as a hydrogen acceptor for the tetrahydro derivative. The oxidized form of the oxygen addition product may then exist either as such or react with cysteine or reduced glutathione and be converted into the dihydrodisulfydryl radical derivative (formula III), the degree of oxidation of the oxygen addition product depending entirely upon the constituents of the solution. The reduced indigo carmine, which is formed, prevents the reducing potential from dropping below the value \(-0.136\)
volts, and it produces a poising effect in the solutions which stabilizes the oxygen addition product.

If the oxygen addition product has any significant bearing on oxidation in the animal organism, some substance other than indigo carmine must serve this purpose. The indispensable poising effect of indigo carmine in our solutions permits the formation of the oxygen addition product in sufficient concentration to demonstrate its catalytic nature and its ability to establish in the solution a reversible oxidation-reduction system.

For many months after the existence of the oxygen addition product had been shown, it was always prepared through the interaction with indigo carmine. Although this procedure gave
satisfactory solutions, it did not explain the reactions involved. Other substances used, in attempts to produce the oxygen addition product in the absence of indigo carmine, were dibromoindophenol, naphthol indodichlorophenol, and methylene blue (Figs. 9 and 10). It was impossible to demonstrate the presence of the oxygen addition product when any one of these three dyes was used in place of indigo carmine. Since all of these substances are rapidly reduced by cysteine, and since the oxygen addition product cannot be formed in the presence of reduced indigo carmine, the failure of the other hydrogen acceptors to produce the oxygen addition product appeared to be because they are so easily reduced. This was shown to be true by mixing the hydrogen dioxide with the dyes and then adding the solution on top of the solution of cysteine and cystine in the electrode chamber without agitation, and without mixing the two layers. After several hours, the electrode chambers were agitated and it was then found that the solutions manifested typical poising action and showed the presence of the oxygen addition product. This was done with dibromoindophenol and naphthol indodichlorophenol. A slight trace only of the oxygen addition product was apparently produced when methylene blue was substituted for indigo carmine.

The effect of the physical state in the formation of the oxygen addition product and the narrow limits within which it is necessary to work suggest that in physiologic processes, cell membranes and the physical condition of the medium are important factors influencing the activation of glutathione and possibly other substances of similar nature (10).

Preliminary experiments have shown that after a suspension of yeast has been boiled in the absence of oxygen, nothing is present in the solution which can reduce indigo carmine. If to such a solution oxygen-free cysteine and cystine are added, there is also no reduction of the dye, but the subsequent addition of a small amount of hydrogen dioxide or molecular oxygen results in an almost immediate reduction of the indigo carmine.

**Apparatus and Materials.**

The apparatus used for this work was similar to that described in an article concerning the oxidation and reduction potentials of 2-oxydihydroindole-3-propionic acid (9). In place of the mercury
Glutathione

seal, however, a copper tube of 6 mm. bore, which permitted the raising and lowering of the electrode chambers without interruption of the flow of nitrogen, was arranged in such a manner that two coils placed in the line of the tubing absorbed any of the strain resulting from raising the electrode chamber. The copper tube was connected with a Pyrex stop-cock and delivery tube. Eight electrode chambers were placed side by side in the thermostat instead of four, and two movable burettes greatly facilitated the addition of the various solutions to the electrode chambers. The temperature of the thermostat was kept at 30° ± 0.1°. The electrode chambers were made out of 10 oz. Hygeia nursing bottles, which fitted into a No. 10 solid rubber stopper. Through the stopper five straight holes with smooth walls were drilled; in four of these holes tubes were placed to admit the nitrogen, to hold the platinum electrode, to connect with the salt bridge into which the calomel electrode dipped, and to give an exit for the nitrogen. The fifth hole was used when solutions were added to the electrode chamber from the movable burettes. This was sealed with a glass rod except when the burette was attached to the electrode chamber. A Leeds and Northrup type K potentiometer was used, and a type G galvanometer. The single electrode potential of the normal hydrogen electrode being designated as zero, the reference standard for this work was a hydrogen electrode in 0.05 M potassium acid phthalate at 30°, which gave a potential difference against saturated calomel electrodes very close to Clark’s value of 0.4827 volts (2). Accordingly the value of -0.244 was assumed for the saturated calomel electrodes used.

The results described in this paper could not have been determined except with an apparatus arranged in such a manner that the color of the solutions in the electrode chambers could be seen at any time desired. Reducing potentials, in relation to color changes, always showed a corresponding value. It was especially desirable to watch the precipitation of indigo from a solution of reduced indigo when it was oxidized with cystine, and it is doubtful whether the chemical properties of the oxygen addition product could have been established without the use of an apparatus which permitted the direct observation of the contents of the electrode chambers whenever it seemed desirable.

When the electrode chamber was raised, contact between the
salt bridge and the cup containing potassium chloride which communicated with the calomel half-cell was interrupted, and during this brief interval it was impossible to determine the reducing potential.

The cystine and cysteine hydrochloride were both prepared in this laboratory and were freed from iron through treatment of their barium salt with hydrogen sulfide (6). The glutathione was prepared from yeast. It was rendered iron-free by the same technique used for cysteine and cystine. The indigo and indigo carmine were commercial samples. The dibromoindophenol and 1-naphthol-2-sulfonate, indo-2,6-dichlorophenol were prepared by E. J. Witzemann after methods indicated by Clark (3). The preparation of reduced indigo has been described (9).

EXPERIMENTAL.

During the investigation of the oxidation-reduction potentials of solutions containing cysteine and cystine, a large number of results have been obtained which it will be impossible to publish in detail. In Table I is given the concentration of all the substances placed in the electrode chambers for the experiments charted in Figs. 1 to 10.

Hydrogen Ion Concentrations of the Solutions.

80 cc. of M/15 disodium phosphate and 20 cc. of M/15 potassium dihydrogen phosphate giving a pH of 7.4 were used in each experiment. To this the cysteine hydrochloride, the sodium salt of cystine, or GSH and GSS solutions, were added and in addition sufficient 0.10 N sodium hydroxide to neutralize all or part of the hydrochloride of cysteine or the acidity of the GSH and GSS solutions. The hydrogen ion of the solutions was maintained the same throughout all of the electrode chambers except in Fig. 8. The difference between the total acid and the total alkali added was 6 cc. of 0.10 N acid in all solutions except those represented in Fig. 8. The total acid was the concentration of cysteine hydrochloride, GSH and GSS, and a small amount of mineral acid in the GSH and GSS; the total alkalinity was the sodium salt of cystine and the added sodium hydroxide. In Fig. 8 there was no excess acid in solutions represented in Charts 2 and 4, and there were
### TABLE I.
Concentrations of Substances in Electrode Chambers.

<table>
<thead>
<tr>
<th>Fig. No.</th>
<th>Chart No.</th>
<th>Cysteine (CSH) or reduced glutathione added.</th>
<th>Cysteine (CSH) or reduced glutathione added.</th>
<th>Ratio of cysteine to cystine dioxide of H₂O₂ and indigo carmine.</th>
<th>Hydrogen dioxide 0.1 N or sodium disulfide 0.01 N.</th>
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E. C. Kendall and F. F. Nord

**TABLE I—Concluded.**

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<thead>
<tr>
<th>Fig. No.</th>
<th>Chart No.</th>
<th>Cysteine (CSH) or reduced glutathione added.</th>
<th>Cysteine (CSH) or oxidized glutathiones added.</th>
<th>Hydrogen dioxide 0.1 N or sodium disulfide 0.01 N.</th>
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12 cc. of 0.10 N acid in the solutions represented in Charts 1 and 3. The change in potential after the addition of 6 cc. of 0.10 N H₂SO₄ is shown in Figs. 3 and 5.

In order to conserve space, the following letters have been used to designate the solutions added to the electrode chambers and procedures followed. C, cc. of 0.10 N hydrogen dioxide. D, cc. of 0.01 N sodium disulfide. E, agitation of the solution was started by the bubbling of oxygen-free nitrogen. F, agitation of solution was stopped by stopping the flow of oxygen-free nitrogen. H, cc. of 0.01 N dibromo indophenol. J, cc. of 0.10 N sulfuric acid. L, cc. of 0.10 N reduced glutathione. M, cc. of 0.0175 N reduced indigo carmine. N, cc. of 0.01 N ferric chloride.

The two solutions most frequently added to the electrode chambers, in order to determine the oxidizing and reducing power of the solutions, were 0.035 N indigo carmine and 0.02 N reduced indigo. In all the figures, all sharp turns in the curves not marked by a letter, or number, are caused by the addition of either indigo carmine or reduced indigo in 1 cc. portions. If more than 1 cc. of reduced indigo was added, the volume in cc. is recorded on the charts by a single number, not followed by a letter, above the line of the curve; all decreases in potential were brought about by the addition of 1 cc. portions of 0.035 N indigo carmine, unless other-
wise noted, by a single number below the line of the curve. The addition and volume of all solutions except indigo carmine and reduced indigo are designated by a number and letter. Wherever uncertainty arises, because of the simultaneous agitation of the solution and the addition of reduced indigo or indigo carmine, a note will be found in the experimental details of the figures.

Experimental Details of Figures.

It has been impossible to place the first part of the curve in all the charts of Figs. 1, 2, 3, and 4. The solutions were prepared by adding the volume of hydrogen dioxide shown in Table I to the electrode chamber and allowing the solution to stand without agitation until the indigo carmine present was reduced. Agitation was then produced by bubbling nitrogen through the solution. Unless the solutions were allowed to stand during the action of the hydrogen dioxide without agitation, no poising action could be demonstrated, unless large volumes of hydrogen dioxide were added.

Fig. 1. 1 cc. of reduced indigo was added (Chart 1) at 2 hours and 50 minutes, and at 22 hours and 12 minutes. All other sharp increases in reducing potentials were caused by the addition of reduced indigo in 1 cc. portions or in the amounts indicated by single number not followed by a letter, above the line of the curve. 1 cc. of reduced indigo was added in Chart 3, at 22 hours and 12 minutes, and in Chart 4, at 22 hours and 15 minutes. The 0.10 N sulfuric acid added in Charts 2, 3, and 4 at about the 26th hour was used to neutralize the alkali added in the solutions of reduced indigo.

Fig. 2. 1 cc. of reduced indigo was added at 22 hours and 24 minutes, in Charts 1, 2, and 3. 2 cc. of indigo carmine were added, Chart 3, at 28 hours and 55 minutes, and 1 cc. of indigo carmine was added to the solution represented by Chart 4 at 30 hours and 36 minutes. The 0.10 N sulfuric acid added at about the 29th hour in Charts 2, 3, and 4 was to neutralize the alkali added in the solution of reduced indigo.

Fig. 3. Chart 1. 6 cc. of 0.10 N sulfuric acid caused the decrease in reducing potential, at 21 hours, 5 minutes. 1 cc. of indigo carmine was added at the 22nd hour in Chart 1, producing the decrease in potential. In Chart 2, 1 cc. of reduced indigo and
not agitation at 2 hours and 10 minutes produced the increase in potential. 6 cc. of 0.10 N sulfuric acid only were added at the 21st hour. In Chart 3, the decrease in reducing potential at 21 hours and 12 minutes was brought about by 6 cc. of 0.10 N sulfuric acid only.

Fig. 4. The change in potential at 2 hours and 12 minutes in Chart 1, was brought about by agitation and the decrease in potential at 21 hours and 5 minutes was caused by the addition of 6 cc. of 0.10 N sulfuric acid alone. In Chart 2, agitation produced a relatively large drop in reducing potential; this was due to the fact that when the solution stands without agitation, the reducing potential varies markedly in different parts of the solution. In Charts 2 and 3, 6 cc. of 0.10 N sulfuric acid caused the drop in potential at 21 hours and 12 minutes.

Fig. 6. Chart 4. The sudden increase at 2 hours and 40 minutes is due to agitation and not the addition of reduced indigo.

Fig. 7. Chart 4. The increase in potential at 2 hours and 40 minutes is due to agitation only.

Fig. 8. All increases in reducing potential were brought about by the addition of 1 cc. of 0.02 reduced indigo, except where the volume added is shown by a single number not followed by a letter.

Fig. 9. Chart 1. The decrease in potential in Chart 1, at 0 hour and 12 minutes, and at 3 hours and 24 minutes, is due to dibromomindophenol. The increase in potential at 15 hours and 40 minutes, and at 40 hours and 20 minutes, is due to agitation alone. Chart 2. The increase in potential between 15 hours and 40 minutes and 18 hours is due to agitation alone. The decrease in potential at the 40th hour, is due to agitation alone.

Fig. 10. Chart 2. The sudden increase in potential at 3 hours and 12 minutes was brought about by 1 cc. of reduced indigo and not by agitation of the solution. 1 cc. of reduced indigo was also added at the 3rd hour in Chart 4, causing the increase.

DISCUSSION.

After the factors influencing the activity of the various constituents had been determined, many of the early results were shown to be of little value. Forty-two typical experiments are given in detail, and negative results will be stated without tabula-
tion or presentation in a chart. The data presented have been chosen to show the following: Figs. 1 and 2, reversible oxidation-reduction in solutions of cysteine and cystine in the presence of the oxygen addition product. Figs. 3 and 4, reversible oxidation-reduction in solutions of reduced and oxidized glutathione in the presence of the oxygen addition product. Fig. 5, the inability of reduced glutathione to reduce indigo carmine completely in the absence of traces of oxygen, hydrogen dioxide, or sodium disulfide. Fig. 6, reversible oxidation-reduction in solutions of cysteine and cystine in the presence of the sulfur addition product. Fig. 7, reversible oxidation-reduction in solutions of reduced and oxidized glutathione in the presence of the sulfur addition product. The oxidation of reduced indigo by sodium disulfide. Fig. 8, Chart 1, the inability of oxidized glutathione to oxidize reduced indigo in the absence of the oxygen addition product; Chart 2, formation of the oxygen addition product from molecular oxygen in a solution containing reduced glutathione and indigo carmine. Fig. 10, the inability of cystine and oxidized glutathione to oxidize reduced indigo, and of cysteine to reduce indigo carmine, even after the addition of sodium disulfide to the solution, if the sulfur addition product is not formed.

Further details of the work also presented in the charts are: The change in the equilibrium point of the solutions, varying with the ratio of cysteine to cystine or GSH to GSS. See Figs. 1 to 4, 6 and 7.

Figs. 1 and 2 show the reversible oxidation-reduction system with cysteine, cystine, and the oxygen addition product. As the concentration of cysteine decreases and that of cystine increases, the oxidation-reduction potential at the equilibrium point of the solution decreases.

The slow oxidation of reduced indigo in the presence of a large amount of cysteine, or GSH, but the rapid oxidation of reduced indigo in the presence of a large amount of cystine or GSS (Figs. 1 to 4, 6, and 7).

The rapid reduction of indigo carmine in the presence of a large amount of cysteine, or GSH, and the slow reduction of indigo carmine in the presence of a large amount of cystine, or GSS (Figs. 1 to 4, 6, and 7).

Increase in Reducing Potential after Addition of Hydrogen Di-
oxide.—The steep slopes in the curves on the charts in Figs. 1 to 5 show the rapid production of a high reducing potential after the addition of the hydrogen dioxide.

The increase in reducing potential after addition of sodium disulfide is shown in Figs. 5 and 6. It has been possible to show the entire change in oxidation-reduction potentials. The velocity of increase in potential when sodium disulfide was added to the solution was of the same order as the velocity of increase of the reducing potential when hydrogen dioxide was used. In Fig. 6, Charts 1 to 4, the addition of sodium disulfide to the solutions of cysteine and cystine did not produce even a momentary decrease in the potential. As the concentration of cysteine diminished, a momentary decrease in the potential became evident. This is shown in Fig. 6, Charts 5 to 8. When sodium disulfide was added to GSH and GSS, however, no decrease in the potential was produced, even in the solutions containing but a small amount of GSH (see Fig. 7).

Constancy of Potential at the Equilibrium Point.—It is to be noted, Fig. 4, Chart 2, that agitation dropped the potential to a value which was practically the equilibrium point of the solution, as shown by subsequent additions of reduced indigo and indigo carmine. Also after the rapid decrease due to agitation, the potential did not change during the following 25 minutes. In Chart 3, agitation produced very little change in reducing potential; the solution appeared to be at its equilibrium point when agitation was started. In Charts 1 to 3, the constant value for the oxidation-reduction potential between 7 hours and 11 hours and 30 minutes is to be noted. This emphasizes the stability of the solutions and the absence of any tendency for the potential to drift after the equilibrium point has been reached.

Effect of the Sulfur Addition Product on Potential of Solutions of Cysteine.—Fig. 7 shows the striking drop in reducing potential produced by the addition of indigo carmine and the increase in reducing potential following the addition of sodium disulfide. A point of great significance which is well illustrated in this figure, is the difference between the reducing potential of these six solutions before the addition of indigo carmine and the equilibrium point after the addition of indigo carmine and sodium disulfide. In Charts 1, 3, 5, and 6, the equilibrium point is distinctly lower
than the potential of the solution before the addition of indigo carmine and sodium disulfide. The change in the potential produced by the oxygen or sulfur addition product has been noted many times, and is explained by the failure of cystine or GSS to affect the platinum electrode. After the oxygen or sulfur addition product has been formed, the equilibrium point of the solution may or may not be the same as before its preparation, depending on whether the solution of cysteine, or of GSH, had reached its equilibrium point.

The decrease in potential when sodium disulfide is added to solutions of cysteine and glutathione, in the absence of indigo carmine, is well shown in Fig. 10. Sodium disulfide added to cysteine or glutathione solutions in the absence of indigo carmine produces a decrease in reducing potential and the addition of reduced indigo is not followed by any poising action. A slow increase in reducing potential may follow the addition of reduced indigo to such a solution, as is shown in Fig. 10, Charts 1 and 4. The addition of indigo carmine to such a solution is not followed by reduction of the indigo carmine. This is shown in Fig. 10, Charts 2 and 4.

The sulfur addition product is not formed in the presence of reduced indigo carmine (Fig. 10, Chart 2). 4 cc. of 0.0175 N reduced indigo carmine were added to the solution just preceding the addition of 10 cc. of 0.01 N sodium disulfide. The sulfur addition product did not form in the presence of the reduced indigo carmine. The influence of indigo carmine on the formation of the sulfur addition product is shown by a comparison of the curves given in Fig. 10 with the curves in Figs. 6 and 7. The only difference in the solutions is the presence of indigo carmine (Figs. 6 and 7) and the absence of indigo carmine (Fig. 10).

The decrease in potential following the addition of sodium disulfide and the failure of these solutions to reduce indigo carmine or oxidize reduced indigo, is striking evidence indicating the necessity of indigo carmine or other hydrogen acceptor in the formation of the sulfur addition product.

GSS cannot oxidize reduced indigo in absence of oxygen addition product. During the first 23 hours, Fig. 9, Chart 2, differs from Chart 1 only in the addition of 10 cc. more of indophenol at 3 hours and 40 minutes. The larger amount of indophenol, however, did not produce any significant change. At 24 hours and 18 min-
utes, 8 cc. more of indigo carmine were added (Chart 2), and at the 27th hour agitation of the solution with nitrogen was stopped, and 10 cc. of the nitrogen in the space above the solution in the electrode chamber were displaced with air. At the 40th hour, the solution was agitated, and a decided drop in reducing potential was produced by agitation alone. This is evidence that on the surface of the solution the indigo carmine was in the oxidized form, and when the solution was agitated, the equilibrium point ultimately reached was markedly lower than that of the solution in contact with the platinum electrode before agitation. It is significant that in Chart 1, the reverse occurred; there was an increase in reducing potential produced by agitation. Following the addition of 3 cc. of reduced indigo to each of these solutions, there was an increase in reducing potential. In Chart 1, no oxidation of the indigo occurred during the succeeding 7 hours, but in Chart 2, the reduced indigo was oxidized by the oxidized glutathione, which was enabled to exert its oxidizing action through the oxygen addition product. 2 cc. more of reduced indigo subsequently added were also oxidized. The results shown in Fig. 9 typify the progress of the work during the early months of this investigation. At that time the necessity of the oxygen addition product was not recognized and the failure to oxidize reduced indigo in the solution represented by Chart 1 was difficult to explain. Convincing evidence that reduced indigo is stable in the presence of oxidized glutathione is shown by the curves in Fig. 9, Charts 1 and 2.

*GSH cannot reduce indigo carmine in the absence of oxygen* (Fig. 5). At the beginning of the experiment Charts 1 and 3 differ from Charts 2 and 4 only in the time at which indigo carmine was added. In Charts 1 and 3, 4 cc. of indigo carmine were present in the electrode chamber, and the addition of 6 cc. of 0.10 N GSH caused a drop in potential, followed by a slow increase during the succeeding 27 hours in Chart 3, and 30 hours in Chart 1. The maximal value in Chart 1 is -0.157 volts, in Chart 3 it is -0.164 volts; however, in Charts 2 and 4, GSH in the absence of indigo carmine produced reducing potential close to -0.27 volts. These results establish the fact that GSHII in the absence of oxygen cannot reduce indigo carmine and produce a high reducing potential. The addition of iron (Charts 3 and 4) did not result in the formation of any substance which could reduce indigo carmine. The
addition of hydrogen dioxide (Charts 1 and 3), produced a decrease and then an increase in potential, which brought the solution back to approximately the same potential. It is to be noted, however, that the equilibrium point reached in all four solutions, was much lower than the potential given by the GSH alone, previous to the addition of indigo carmine (Charts 3 and 4). The addition of hydrogen dioxide (Charts 2 and 4) and of sodium disulfide (Charts 1 and 3) caused a reduction of the indigo carmine in each case, so that the color of the solution at the equilibrium point was that of almost completely reduced indigo carmine. In order to obtain solutions of GSH so inactive with indigo carmine, it is necessary to deoxygenate the buffers and GSH solutions for many hours. Unless this is done, the GSH rapidly reduces indigo carmine.

The influence of iron is shown in Fig. 5, Charts 3 and 4. 5 cc. of 0.01 N ferric chloride caused a drop in the reducing potential, followed by a slow increase. The presence of iron in the solution did not activate the cysteine, so that the indigo carmine was reduced, although the subsequent addition of hydrogen dioxide brought about a rapid reduction of the indigo carmine.

No chart or table giving the details of the following experiments will be published. The results were carried out in a manner entirely similar to the experiments described in detail.

The oxygen addition product cannot be formed with hydrogen dioxide in the presence of reduced indigo carmine. In solutions prepared in identically the same manner as shown in Figs. 1 to 5, no oxidation of reduced indigo could be demonstrated if reduced indigo carmine was present in the solutions when hydrogen dioxide was added.

No oxygen addition product is formed with hydrogen dioxide and cysteine alone, in the absence of indigo carmine or other hydrogen acceptor. If any hydrogen acceptor other than indigo carmine is used, the results are variable, and often negative. The best conditions for the use of other hydrogen acceptors is to mix the hydrogen dioxide with the oxidizing dye and add the solution to the electrode chamber without agitation, so that a layer is formed over the solution. After this has stood for several hours, it is gently agitated. Solutions prepared in this way will reduce indigo carmine and will oxidize reduced indigo.

Changing Potentials in Solutions of Cysteine.—The slow drift
in the potential with solutions containing cysteine, at pH 7.4, may continue for 5 or 6 hours. The value eventually reached appears to be of significance, as it can be duplicated. The addition of a small amount of indigo carmine to such a solution causes a drop in the potential approximately to $-0.136$ volts. In the presence of indigo carmine, the potential does not again reach its former high value and will remain at about the value $-0.136$ for many hours. If, however, hydrogen dioxide, naphthol indodiclorophenol, dibromoinophenol, or methylene blue are added to the solution instead of indigo carmine, there is a drop in potential, which is followed by an increase to the former high value. Indigo carmine is the only oxidizing dye which we have investigated which is not reduced by cysteine.

**SUMMARY.**

Dixon and Quastel showed that in solutions of cysteine and cystine "continuous and extensive drifts of the potential appear to form the main feature of the system if the ordinary platinum calomel cell be adopted." To overcome the experimental difficulties in working with those substances, they suggest the use of a gold electrode. Our results confirm those of Dixon and Quastel concerning the drift in the potential, but it seems more probable that the cause for the drift is in changes occurring in the sulfydryl group rather than in the influence of the electrode. We have been able to obtain solutions in which all drift has been eliminated and which gave values that were reproducible by merely allowing the solutions to stand several hours until equilibrium was reached. The probable change in the sulfydryl group causing the drift in the potential is a combination of two molecules of cysteine forming a compound for which the following formula is suggested.

\[
\begin{align*}
\text{H} \\
\text{C - S - H} \\
\text{C - S}
\end{align*}
\]

Dixon and Quastel also showed that cystine has but little effect on the platinum or gold electrode and that the reducing potential varies with the concentration of the cysteine. Our results confirm
their conclusion that cystine does not affect the platinum electrode. We have furthermore shown that cystine will not oxidize reduced indigo. However, the equation which they suggest for the relation between the reducing potential and the concentration of cysteine cannot be used as an expression of the activity of the system under all conditions. We have found that in the presence of indigo carmine, or other hydrogen acceptor, hydrogen dioxide and sodium disulfide will react with cysteine forming an addition product. In solutions containing cysteine and cystine or GSH and GSS with the oxygen or sulfur addition product, the potential does not vary with the concentration of cysteine alone, but the ratio of cysteine to cystine determines the absolute value of the oxidation-reduction potential at the equilibrium point. In such a solution reduced indigo is rapidly oxidized, and indigo carmine is rapidly reduced. In the absence of the oxygen addition product, cysteine cannot reduce indigo carmine, and cystine cannot oxidize reduced indigo.

It is also possible to deoxygenate a solution of reduced glutathione so that it cannot reduce indigo carmine. If molecular oxygen, hydrogen dioxide, or sodium disulfide is added to such a solution, the indigo carmine is almost immediately reduced; it is furthermore found that solutions thus prepared form a reversible oxidation-reduction system.

The investigations of Thunberg, Meyerhof, Hopkins, Dixon, Tunnicliffe, and others have shown that the SS grouping can be reduced with sulfhydryl groups and can catalyze oxidation in boiled muscle and liver preparations. The results recorded in this paper extend these observations and establish the conditions necessary and sufficient to form a reversible oxidation-reduction system with reduced and oxidized glutathione. The essential intermediate compound of such a reversible system is an oxygen addition product of reduced glutathione. Our results further indicate that the reduced and oxidized forms of glutathione are relatively stable substances in which the atom of sulfur cannot change its state of oxidation with sufficient ease to influence physiological processes of oxidation and reduction. Under certain narrow but definite conditions, glutathione can exist in the form of a highly reactive oxygen addition product in which the atom of sulfur changes its state of oxidation with every addition of suitable oxidizing and reducing substances to the solution. The more
stable SH and SS forms of glutathione can react with the oxygen addition product, and the three forms of this compound make a reversible oxidation-reduction system. The chemical properties of the oxygen addition product of glutathione indicate that it is the sine qua non of the activity of glutathione in physiological processes of oxidation in so far as a reversible system is concerned.

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REVERSIBLE
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