CXIV. ON THE REDUCING POWER OF GLUTATHIONE AND CYSTEINE.

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KENDALL and NORD [1926] in a recent paper have put forward a somewhat novel view of the mechanism of reduction by —SH groups. They state that, in the absence of oxygen, cysteine and reduced glutathione are unable to reduce indigo carmine, but that in the presence of small amounts of oxygen an active addition compound of the —SH group with oxygen is formed, whereby the system acquires the power to reduce the dye. The same "activation" was obtained by the addition of hydrogen peroxide or of sodium disulphide.

In view of the importance of such a fact in considering the functions of sulphur compounds in cell respiration processes, the experimental basis for such a view calls for careful consideration.

THE REDUCING POWER OF CYSTEINE AND GLUTATHIONE IN THE ABSENCE OF OXYGEN.

Kendall and Nord placed solutions of indigo carmine and cysteine in phosphate buffer in a vessel through which nitrogen was bubbled continuously. After 40 hours at 30° the solutions still remained deep blue, from which they concluded that no reduction of the dye had taken place. This result was at variance with some unpublished observations made by us in 1922, in which, using the Thunberg vacuum-tube technique, rapid reduction of indigo carmine by cysteine was found to occur. It seemed to us that the discrepancy might be due to the presence of traces of oxygen, particularly in view of experience of the difficulty in obtaining nitrogen in quantity free from such traces.

In view of the large volume of gas which Kendall and Nord must have passed through their solution it will be clear that a very small percentage of oxygen in the nitrogen might have been sufficient to prevent decoloration, owing to the great ease with which reduced indigo carmine is reoxidised by oxygen. Assuming (information on this point being absent from Kendall and Nord's paper) a rate of bubbling of 1 cc. per second, then in 40 hours 144 litres of nitrogen would flow. Even if the gas were 99.99 % pure this would mean that nearly 15 cc. of oxygen would pass into the solution during the experiment, a quantity sufficient to reoxidise the whole of the indigo carmine many times over.

In order to decide whether cysteine can reduce indigo carmine in the absence of oxygen we made experiments, taking extreme precautions to remove all traces of oxygen. For this purpose the special vacuum-tube represented in Fig. 1 in front and side view was employed.

Fig. 1.

The open end *D* was provided with a well-fitting rubber stopper carrying a glass tube with a tap, so that the tube could be evacuated. The neutral cysteine solution (previously kept in an exhausted vacuum-tube for 2 or 3 days) was placed with an appropriate volume of buffer solution (p_H 7.6) in the limb *A*, and the indigo carmine solution in *B*. A strong solution of sodium hydrosulphite in water and caustic soda were placed in the bulb *C* by means of a special curved pipette, and the apparatus immediately exhausted by an efficient water-pump. When no further bubbles of dissolved gas could be obtained from the liquids, nitrogen was admitted and the apparatus re-exhausted. The process was again repeated, the tap closed after the final exhaustion and the apparatus allowed to stand for 24 hours or more with gentle shaking at intervals. At the end of this period the solutions in *A* and *B* were carefully mixed by tilting the tube, and the apparatus was placed in the bath at 40°. (The apparatus was so constructed and handled that there was no possibility of any trace of the hydrosulphite getting into the reacting solutions. In no case have we observed the slightest reduction of the dye before the solutions were mixed, or in control experiments without the —SH compound.) In all cases rapid and complete reduction of the indigo carmine occurred. Using 0.3 cc. indigo carmine (0.035 *M*) + 1.0 cc. cysteine (0.05 *M*) + 3 cc. phosphate buffer the dye was completely reduced in 6 minutes. At the end of the experiment the vacuum in the tube was tested by immersing the tube in water and opening the tap. The water filled the tube with the exception of a small bubble of approximately 50 mm.³. Assuming that our nitrogen contained as much as 1 % of oxygen it will be clear that the maximum possible quantity of oxygen in the tube at the end of the final exhaustion is 0.5 mm.³, and this will of course be rapidly and completely absorbed by the hydrosulphite solution, which as is well known is an extremely efficient deoxygenating agent.

In a series of experiments we found that the more carefully traces of oxygen are removed, the smaller becomes the time for reduction of the dye (provided that large amounts of oxygen are not present; cf. the following section).

Similar results have been obtained with reduced glutathione.

The preparations of cysteine used by us, though very pure, were not absolutely iron-free in Warburg's sense, although the fact that no trace of colour developed in the solutions on making faintly alkaline with ammonia and shaking with oxygen shows that this was very nearly the case. Kendall and Nord freed their cysteine preparations from iron by the Warburg and Sakuma [1923] technique; but the effect of this was largely negated by the fact that they used, without purification, commercial indigo carmine, which would certainly contain small amounts of iron. In view of the recent work of Harrison [1927], who found that the reduction of methylene blue by cysteine was largely (but not entirely) due to catalysis by traces of iron, it became necessary to consider how far the reduction of indigo carmine observed by us was due to the same cause. We obtained with indigo carmine results very similar to those obtained by Harrison with methylene blue. The addition of very small traces of ferric chloride produced a marked acceleration of the reduction, while the addition of a small amount of KCN slowed the reaction considerably. But, as is the case with methylene blue, even when the metal-catalysis is completely abolished by the addition of KCN, and when all traces of oxygen are removed, the reaction still proceeds with a quite considerable velocity.

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. As this contention is fundamental to their theory, we propose to discuss the bearing of the facts given above on their evidence for the formation of an oxygen addition compound.

When Kendall and Nord stopped the flow of gas through their solutions, and allowed them to stand undisturbed for some hours, they observed that occasionally the dye became reduced, with the exception of a blue layer on the surface of the liquid. Kendall and Nord concluded that in these cases oxygen had leaked into the apparatus, and by its presence had enabled the reduction to be brought about, owing to some activating property. In order to test this hypothesis they proceeded as follows. After bubbling nitrogen through the mixture of cysteine and indigo carmine for several hours, with no apparent reduction of the dye, the bubbling was stopped and 10 cc. of air admitted. On allowing to stand undisturbed for 2 hours, reduction was complete in a layer 2 cm. below the surface.

A possible interpretation of this experiment seems to be that the traces of oxygen continuously introduced by the nitrogen maintained the dye in its oxidised form. On cessation of bubbling the rate of diffusion of oxygen into the deeper layers of the solution is not sufficient to mask the effect of the reduction by the —SH compound. There therefore results a colourless deep layer and a blue surface layer as Kendall and Nord observed.

This conclusion is strikingly supported by the following internal evidence in Kendall and Nord's account.

(1) "The reduction was retarded in proportion to the agitation," *i.e.* to the volume of gas passing through the solution.

(2) "When the air was mixed with the nitrogen and bubbled through the solution, little or no reduction of the indigo carmine occurred"; this is a very significant statement, since there seems no reason on their view why oxygen leaking into the apparatus should be able to cause the formation of the addition product, while oxygen deliberately introduced with the nitrogen should be unable to act. In order to account for this result Kendall and Nord were obliged to assume that agitation prevents the formation of the oxygen addition product!

In view of the above facts, which would appear to invalidate Kendall and Nord's experiments, we considered it necessary to reinvestigate the question whether oxygen can *accelerate* the reduction of indigo carmine. In the presence of fairly large amounts of oxygen we have been able to observe such an acceleration. For instance, if a neutral solution containing cysteine and indigo carmine is divided into two portions, one being placed in an evacuated Thunberg tube and the other in an open test-tube, it will be found that reduction is complete in the open tube (except of course at the surface of the liquid) definitely sooner than in the vacuum-tube.

We are of the opinion that this phenomenon is due to the hydrogen peroxide formed during the autoxidation of the —SH group [Thurlow, 1925], and we discuss its interpretation more fully in the following section of the paper.

THE RÔLE OF HYDROGEN PEROXIDE IN THE FORMATION OF THE ADDITION COMPOUND.

Kendall and Nord state that hydrogen dioxide is also able to form an active addition compound: "hydrogen dioxide was therefore added to solutions of cysteine and after some time indigo carmine added. No reduction 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." Kendall and Nord consider that these results indicate the existence of an essential intermediate compound which is formed by the action of hydrogen dioxide on cysteine.

We have confirmed these observations, but we do not agree with this interpretation of the phenomenon. It is obvious that the action of the hydrogen peroxide may be either on the cysteine or on the indigo carmine. The fact that according to Kendall and Nord the oxygen addition compound is only formed when indigo carmine is present, for which they offer no satisfactory explanation, suggests that the active compound may be due to the action of the peroxide on the dye rather than on the cysteine. It is well known that hydrogen per-

oxide reacts readily with indigo carmine, and with dyes on which hydrogen peroxide has no action Kendall and Nord failed to obtain effects comparable with those observed with indigo carmine. We have found for instance that hydrogen peroxide produces no acceleration of the reduction of methylene blue by cysteine.

In order to test the possibility that a catalytically active compound is formed from the indigo carmine we carried out the following experiments.

A solution of indigo carmine in phosphate buffer p_H 7.6 was treated with dilute hydrogen peroxide, and the mixture allowed to react until *slight* bleaching had occurred. Manganese dioxide was then added, the solution warmed to decompose all traces of H_2O_2 , and the mixture filtered (solution *A*). A second solution of untreated indigo carmine was then prepared (solution *B*) of the same depth of colour as solution *A*. The two solutions were thus identical, except that *A* contained a small amount of the oxidation-product of indigo carmine. Equal volumes of the two solutions were placed separately in two Thunberg tubes, equal amounts of cysteine solution added to each, and both tubes thoroughly evacuated. It was found that the treated indigo carmine solution (solution *A*) was reduced with very great rapidity compared with solution *B*, the acceleration produced being comparable with that produced by the direct addition of hydrogen peroxide. That a true reduction, and not merely a bleaching, had occurred was shown by the ready reappearance of the colour on the admission of oxygen. A control was carried out, to make certain that all traces of peroxide had been removed, by adding manganese dioxide to dilute hydrogen peroxide of the same strength as previously and treating in exactly the same way as before. The filtrate gave no test with KI and starch, and on addition to a mixture of indigo carmine and cysteine no accelerating effect could be observed. A further control, in which indigo carmine was treated with MnO_2 without H_2O_2 , gave no acceleration; showing that the acceleration previously observed was not due to catalytic action by traces of manganese.

The result described 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. We consider that this fact provides the true explanation of the phenomena observed by Kendall and Nord, and renders unnecessary the assumption of the formation of an active oxygen addition product with cysteine.

The fact that no effect was observed by Kendall and Nord when the hydrogen peroxide was added to the cysteine, and indigo carmine added only after some time, is clearly due to the removal of the H_2O_2 by the usual reaction with the —SH group before the addition of the indigo carmine. By reduction-potential measurements we have found that under the above conditions, removal of the peroxide is complete in a few minutes.

Hydrogen peroxide (as is so frequently the case) probably requires traces of iron as a catalyst for its action on the indigo carmine. We have found that

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minute traces of ferric or ferrous salts have a marked accelerating effect on the bleaching of indigo carmine by H_2O_2 ; and we have shown that the products of the reaction are active accelerators. Moreover, we have found that cyanide definitely inhibits this reaction. Now if our interpretation be the correct one we should expect to find that the addition of a small amount of KCN, by preventing the action of the peroxide on the dye, would abolish the accelerating effect of the H_2O_2 . This is precisely what occurs, as the following experiment shows.

Contents of tubes					Reduction- time at 35°
Indigo carmine	+	buffer	+	cysteine	1 hr.
"	+	"	+	" + H_2O_2	17 mins.
"	+	"	+	" + " + KCN	3 hrs.
"	+	"	+	" + KCN	4 hrs.

The quantities used were:

Indigo carmine: 1 cc. of 0.035 *M*.

Cysteine: 1.5 cc. of 0.1 *M*.

H_2O_2 : 0.5 cc. of 0.1 *M*.

KCN: final concentration 0.005 *M*.

Phosphate buffer: 3 cc. $p_{\text{H}} = 7.6$.

It will be seen that the presence of the cyanide completely prevents the acceleration by the peroxide, although the normal reduction of the indigo carmine by the cysteine still occurs, as mentioned above.

We conclude from these facts that Kendall and Nord's main phenomenon is dependent on the presence of traces of iron present as impurities, perhaps as simple iron salts, perhaps as a cysteine-iron complex.

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

Kendall and Nord state that addition of 0.01 *N* sodium disulphide solution to cysteine in the presence of indigo carmine brought about the prompt reduction of the dye, and they claim that "cysteine reacts with sodium disulphide in the same manner as it does with hydrogen dioxide."

No information is given as to the method of preparation of the disulphide solution. As ordinarily prepared, by dissolving the theoretical amount of sulphur in a solution of Na_2S , the solution is a most powerful reducing agent, and we have found that it reduces indigo carmine with remarkable rapidity. Kendall and Nord, in fact, also observed this reducing power.

It therefore seems, to say the least, inadvisable to draw such a definite conclusion as that of Kendall and Nord, since the reduction of the dye may be due to the sodium disulphide and not to the cysteine. Kendall and Nord do not appear to have carried out control experiments with sodium disulphide alone.

REDUCTION-POTENTIAL MEASUREMENTS.

Dixon and Quastel [1923] pointed out the unsuitability of the platinum electrode for work on the —SH system, owing to the extensive potential drifts which are met with using this electrode. Kendall and Nord nevertheless carried out all their work with this electrode. They "obtained systems in which the drifts had been eliminated" by simply waiting until the drifts had ceased, and taking the final reading. The significance of these values seems somewhat doubtful.

Furthermore, the hydrogen ion concentrations of Kendall and Nord's solutions were not accurately controlled, and moreover were undetermined. The additions made at various times to their electrode vessels consisted of such solutions as those of cysteine hydrochloride, the sodium salt of cystine, sodium disulphide, and un-neutralised glutathione, together with "sufficient $N/10$ sodium hydroxide to neutralise *all or part* of the acidity" (our italics).

Their method of preparation of solutions of reduced indigo, which they used in experiments on the reversibility of the —SH, —SS— system, seems also to call for some notice [see Kendall and Ort, 1926]. The indigo was reduced by boiling with glucose and sodium hydroxide; and the whole of the resulting mixture of reduced indigo, alkali, and the decomposition products of glucose was used for addition to the electrode vessel, and simply referred to as "reduced indigo" by Kendall and Nord. It will be obvious that such a solution, containing one of the most powerful reducing agents—glucose in the presence of alkali—together with the unknown decomposition products of glucose, will give reduction-potential readings of little significance.

Kendall and Nord state that neutralisation of the above solution was unnecessary, as on oxidising the reduced indigo and filtering off the indigo the resulting solution was neutral to phenol red. We have found, however, that this indicator cannot be used to determine the p_H of such a solution, as on addition of phenol red to a solution of glucose and NaOH which has been boiled, the indicator always assumes a colour approximately corresponding to p_H 7.2. Addition of 40 % caustic soda fails to alter this colour, so that this indicator gives no information as to the p_H of such solutions.

In order to ascertain as far as possible the actual p_H of the solution used by Kendall and Nord we prepared a solution of reduced indigo by their procedure, using the exact quantities given in their paper. After reoxidising and filtering off the indigo the filtrate was found to be alkaline to alizarin (which behaves normally in such solutions); and its p_H must therefore have been at least 12, although it appeared to be neutral to phenol red. With phenolphthalein the maximum red colour was first produced, but this colour vanished almost instantaneously, owing to the reducing properties of the solution.

One of the main points of Kendall and Nord's paper was that in presence of the oxygen addition compound the reduction-potential was determined by the ratio of the concentrations of the —SH compound to the —SS— com-

pound, *i.e.* there was a truly reversible system. Examination of their experimental evidence for this view fails however to establish their conclusion.

Taking a constant amount of cysteine in four electrode vessels, and oxidising varying proportions to cystine by adding different quantities of hydrogen peroxide in the presence of indigo carmine, Kendall and Nord found that the reduction-potential was highest where the ratio $\text{—SH} : \text{—SS—}$ was highest, and varied directly with this ratio. Without further discussion they state that "it is therefore evident that the ratio of cystein to cystin establishes the equilibrium point in these solutions."

Since Dixon and Quastel found that the potential was determined solely by the concentration of the —SH compound, and that the —SS— group is without effect, it is obvious that varying the —SH concentration in the manner described by Kendall and Nord will result in variations of the reduction-potential in the sense observed by them. Any conclusion as to the importance of the *ratio* is fallacious unless the concentration of the —SH group is maintained constant and the concentration of the —SS— group varied, an experiment of which Kendall and Nord make no mention. Kendall and Nord themselves state that the —SS— form does not affect the electrode, thus showing clearly that the potential is determined solely by the —SH concentration and not by the ratio of this to the —SS— . The results obtained by Dixon and Quastel adequately explain the above observations.

Thus it appears that Kendall and Nord have brought forward no evidence of the reversibility of the —SH , —SS— system.

We do not propose to attempt any detailed discussion of the elaborate theories put forward by Kendall and Nord to account for the apparent properties of the hypothetical addition compound. We have confined ourselves in this paper to a consideration of the main points on which these theories are based. In any case it is sure that their results can have but limited application, since they refer only to the reduction of the particular dye indigo carmine, and do not occur with other dyes such as methylene blue. Still less could they have significance in tissue oxidation processes. The claim of Kendall and Nord that the active addition compound of glutathione with oxygen is the *sine qua non* for its physiological activity is not justified by the evidence.

SUMMARY.

There is no necessity for Kendall and Nord's assumption of an active addition compound of the —SH group with oxygen.

Their experiments do not show that the —SH , —SS— system is reversible.

REFERENCES.

- Dixon and Quastel (1923). *J. Chem. Soc.* **123**, 2943.
 Harrison (1927). *Biochem. J.* **21**, 335.
 Kendall and Nord (1926) *J. Biol. Chem.* **69**, 295.
 Kendall and Ort (1926). *J. Biol. Chem.* **68**, 611.
 Thurlow (1925). *Biochem. J.* **19**, 175.
 Warburg and Sakuma (1923). *Pflüger's Arch.* **200**, 203.