XLVII. THE CATALYTIC ACTION OF TRACES OF IRON AND COPPER ON THE ANAEROBIC OXIDATION OF SULPHYDRYL COMPOUNDS.

BY DOUGLAS CREESE HARRISON.

*From the Biochemical Laboratory, Cambridge University, and
the Department of Pharmacology, Sheffield University.*

(Received February 20th, 1927.)

IN a previous publication by the author [1924], it was shown that the rate of oxidation of glutathione and cysteine by the oxygen of the air was greatly reduced by the addition of traces of hydrogen cyanide. It was shown also that the rate of oxidation of glutathione and cysteine could be reduced to a similar low value by very careful purification in quartz vessels, taking all possible precautions to exclude iron; and further, that the rate of oxidation of the purified compounds could be accelerated by the addition of minute traces of iron. Evidence was given in support of Warburg's view that the inhibition by cyanide is due to the formation of a non-catalytic complex with traces of catalytic metals ordinarily present as impurities in the sulphydryl compounds.

During the course of these experiments, I found that not only the aerobic but also the anaerobic oxidation of sulphydryl compounds was greatly inhibited by small concentrations of hydrogen cyanide. In these anaerobic oxidations methylene blue acted as the hydrogen acceptor in place of oxygen. It seemed possible that, as in the case of the aerobic oxidation, the cyanide might be acting on traces of catalytic metal impurities. If this were true, these metals would be expected to catalyse not only the aerobic but also the anaerobic oxidation of sulphydryl compounds, and could not be regarded as acting as carriers or activators of oxygen as pictured by Warburg [1924]. It was therefore desirable to obtain samples of sulphydryl compounds and methylene blue as free as possible from traces of catalytic metals, and then to show that the rate of anaerobic oxidation could be accelerated by the addition of traces of iron to the purified compounds.

The present paper contains the results of these experiments. Most of these results were obtained in 1924, and a brief reference to them was made in a paper by Thurlow [1925]. In 1926, a paper appeared by Toda [1926], containing experiments similar to some of those which I am about to describe.

Both his and my own experiments yielded a similar result, and he draws the same conclusion as was indicated in the reference to my experiments by Thurlow [1925].

Inhibition by hydrogen cyanide.

The following experiments show the great inhibition produced by traces of hydrogen cyanide on the reduction of methylene blue by cysteine, glutathione and thioglycollic acid. In this group of experiments, the vacuum tubes and technique described by Thunberg were used. After twice evacuating and filling with nitrogen, the tubes were again evacuated and immersed in a water-bath, the latter being mechanically stirred and maintained at 37°.

The vacuum tubes each contained 0.3 cc. of methylene blue (1 part in 5000 of water) and a solution of the particular sulphydryl compound neutralised with dilute sodium hydroxide. 0.1 cc. of potassium cyanide solution (previously neutralised with HCl) was added to one tube and an equal volume of water to the other tube. The total volume of solution in each tube was made up to 2.0 cc. with phosphate buffer.

Table I.

Sulphydryl compound	p_H	Time in min. for decoloration of Mb
Cysteine (0.3 mg.) ...	7.0	Without cyanide 12 min.
"	"	+ $M/4000$ HCN 42
"	7.0	Without cyanide 12
"	"	+ $M/8000$ HCN 27
"	7.4	Without cyanide 12
"	"	+ $M/2000$ HCN 45
"	7.6	Without cyanide 10
"	"	+ $M/2000$ HCN 60
Glutathione (1.2 mg.) ...	7.0	Without cyanide 35
"	"	+ $M/2000$ HCN 110
Thioglycollic acid (1.2 mg.)	7.4	Without cyanide 95
"	"	+ $M/4000$ HCN 240

From these experiments it is evident that the addition of very small quantities of cyanide produces a marked inhibition in the rate of reduction of methylene blue by sulphydryl compounds.

On adding iron to solutions containing cyanide, it was found that the inhibition produced by the latter was considerably reduced. On adding iron to the solutions of sulphydryl compounds in the absence of cyanide, the rate of reduction was increased, though this increase was not very marked, owing to the relatively large amount of iron already present in the solutions.

Preparation of iron-free methylene blue.

The method used for preparing iron-free methylene blue was similar to that previously employed by the author in purifying cysteine [Harrison, 1924]. It depends, firstly, on the precipitation of the iron by hydrogen sulphide in presence of baryta, and, secondly, on crystallisation of the leuco-compound from pure alcohol. In view of the fact that cysteine forms a compound with

iron while methylene blue shows no such tendency, it seemed likely that the method would give even better results than with cysteine.

Two grams of crystalline methylene blue (the free base) were added to 150 cc. of water in a Pyrex conical flask which had been previously boiled out with pure concentrated hydrochloric acid. 20 g. of finely powdered crystalline baryta were then added and, after shaking, a rapid stream of well-washed hydrogen sulphide was passed for half an hour. The methylene blue was reduced to the leuco-compound which, together with the iron, was thrown down as a greyish precipitate. This was allowed to stand for 24 hours in a closed vessel, and then shaken with 100 cc. of moist ether which had been previously distilled from quartz vessels and saturated with hydrogen sulphide. The ether dissolves most of the leuco-compound, leaving the iron behind as a greenish basic sulphide. After standing for 2 hours the greater part of the ether layer was carefully poured into a quartz flask, shaken with a few cc. of pure water to remove any traces of barium, and poured off into an acid-extracted filter paper resting in the neck of a quartz flask. After filtering, the mouth of the flask was covered with a filter paper and the ether was distilled off, leaving the pale yellow leuco-methylene blue. This was dissolved in the minimum quantity of hot alcohol (distilled from quartz and containing a little dissolved hydrogen sulphide), half the volume of pure water was added and the solution was cooled under the tap for a short time.

Crystalline leuco-methylene blue separated out in fine needles and was filtered in a quartz Gooch crucible lined with an acid-extracted filter paper, and washed several times with 70 % alcohol (which had been distilled in quartz). If care be taken in the preceding operation to avoid undue exposure to the air, the leuco-methylene blue is obtained quite free from the oxidised form. After drying on the pump for a few minutes, the leuco-compound started to oxidise rapidly. The drying was completed in a vacuum desiccator over sulphuric acid.

The yield obtained under these conditions is small, but may be increased, though at the possible sacrifice of purity, by crystallising from more dilute alcohol.

Purification of sulphhydryl compounds.

Cysteine hydrochloride was rendered free from traces of iron by the method employed in experiments on the aerobic oxidation of cysteine by Sakuma [1923] and Harrison [1924].

Thioglycollic acid was purified from traces of metallic impurities by distillation in hydrogen at 16 mm. Hg pressure, using quartz vessels.

A large number of experiments was then carried out on the rate of reduction of the pure methylene blue by the pure sulphhydryl compounds. Preliminary experiments were carried out in quartz vacuum tubes, but it was found difficult to obtain good agreement between duplicates, possibly owing to the presence of oxygen adsorbed on the walls of the quartz tubes. It was

found, however, that by subjecting glass vacuum tubes to a thorough cleaning with acids before each experiment, contamination of the solutions by traces of catalytic metals did not occur, and that the difficulties associated with the use of quartz vacuum tubes could be obviated.

Owing to variations in the minute quantities of metals remaining in different samples of the purified compounds and to the difficulty of ensuring that absolutely no metallic impurities are introduced during the making up of the solutions, the reduction times observed in different experiments employing the same quantities of sulphhydryl compound and methylene blue varied somewhat. Another possible source of this variation may be the differences in the intensity of illumination of the water-bath on different days. Preliminary experiments appear to indicate that sunlight accelerates the rate of reduction of methylene blue by cysteine and thioglycollic acid, and Dixon and Tunncliffe [1923] observed a similar effect in the case of glutathione. Nevertheless, these facts do not in any way affect the conclusions drawn in this paper on the relative rates of anaerobic oxidation of sulphhydryl compounds in the presence and absence of catalytic metals, for in every experiment duplicate tubes were employed and these duplicates almost invariably showed good agreement. In the few cases where good agreement was not obtained, the results were discarded and the experiments repeated.

Before each experiment, the glass tubes were treated with small quantities of a mixture of concentrated nitric and sulphuric acids to which a few drops of alcohol were cautiously added. After repeating this treatment, the tubes were boiled out with pure concentrated hydrochloric acid and allowed to stand filled with concentrated hydrochloric acid. Finally the tubes were well rinsed out with quartz-distilled water. After this treatment the tubes were found to be perfectly satisfactory and to give good duplicates. The lower part of the ground glass stoppers was left ungreaed to avoid possible contamination of the solution from the tap grease. After dissolving the pure sulphhydryl compound in quartz-distilled water, the solutions were brought to the required p_H by the addition of dilute ammonium hydroxide previously distilled in quartz vessels. The alkali was added from a quartz burette, the volume required being determined by separate titration. No buffer was used in the experiments with the purified compounds. The tubes were evacuated with the water-pump and filled with nitrogen (washed with alkaline pyrogallol). This was repeated, and finally the tubes were evacuated and completely immersed in the water-bath, the side tubes being first filled with water to reduce the chance of leakage. The p_H was checked at the end of each experiment by means of Clark and Lubs' standard indicators. A few drops of the indicator previously boiled to expel any dissolved air were introduced into the side tube of each reduction tube and allowed to mix with the solution inside the tube by partially turning the stopper, care being taken to prevent the entry of any air. In all experiments described in this paper, 0.3 cc. of purified methylene blue (1 part dissolved in 5000 parts of quartz-distilled water) was used in each tube. The total volume

of solution in each tube was always made up to 2.0 cc. In all experiments employing iron, a freshly prepared solution of ferric chloride was used. The copper used was added as copper sulphate solution. The results quoted in the following tables are those of typical experiments chosen from a large number. Each experiment was carried out in duplicate.

Table II shows the catalytic effect of traces of iron on the anaerobic oxidation of purified cysteine at 37°.

Table II.

Each tube contained 0.4 mg. of purified cysteine hydrochloride.
Iron added as ferric chloride. Temp. 37°.

No.	p _H	Iron added (mg.)	Time in min. for decoloration of Mb
1	7.3	—	18 min.
	7.3	0.01	9
2	7.0	—	53
	7.0	0.001	24
3	7.7	—	23
	7.7	0.001	15

It was found that by carrying out the experiments at 25° instead of at 37° the rate of decoloration of methylene blue was slower, and that consequently differences in reduction time could be measured more easily. All the experiments subsequently to be described were therefore carried out at 25°. Table III shows the catalytic effect of traces of iron on the anaerobic oxidation of purified cysteine at 25°.

Table III.

Iron added as FeCl₃. Temp. 25°.

No.	Cysteine-HCl mg.	p _H	Iron added mg.	Reduction time of Mb in min.
1	0.4	8.5	—	90
	"	"	0.001	55
2	0.5	8.4	—	39
	"	"	0.001	18
3	0.8	7.8	—	98
	"	"	0.001	33
4	0.8	7.6	—	50
	"	"	0.001	14
5	0.8	5.6	—	73
	"	"	0.001	57

Table IV.

Each tube contained 0.8 mg. of purified cysteine hydrochloride.
Copper added as CuSO₄. Temp. 25°.

No.	p _H	Cu added mg.	Reduction time of Mb in min.	Reduction time of Mb with HCN
1	7.6	—	50	—
	"	0.0001	9	—
	"	0.001	< ½	—
2	7.4	—	66	+ M/1000 HCN 65 min.
	"	0.0001	8	—
	"	0.0002	6	—
3	7.6	—	60	+ M/500 HCN 72 min.
	"	0.00001	26	—
	"	0.00005	9	—

It is evident from Tables II and III that iron acts as a powerful catalyst in the anaerobic oxidation of cysteine, both on the acid and alkaline side of neutrality.

Table IV shows the very powerful catalytic effect of minute traces of copper on the anaerobic oxidation of purified cysteine.

For purposes of comparison, it may be mentioned that a sample of ordinary unpurified cysteine hydrochloride (0.8 mg.) in p_H 7.6 phosphate buffer under the same experimental conditions as in Table IV reduced the methylene blue in 10 minutes in the absence of cyanide, while in presence of $M/1000$ HCN, it reduced in 70 minutes; on the other hand it will be seen from *Exp.* 3 in Table IV that under similar conditions 0.8 mg. of *purified* cysteine hydrochloride reduced the methylene blue in 60 minutes without HCN, and in 72 minutes after the addition of $M/500$ HCN. From Table IV there is seen to be very little, if any, inhibition produced by HCN on the rate of oxidation of the purified cysteine. From the results in Table IV it is evident that a quantity of copper so minute as five millionths of a milligram per cc. produces a considerable acceleration in the anaerobic oxidation of cysteine. By comparison with Table III, it will be seen that iron is considerably less active as a catalyst than copper. Nevertheless, five ten-thousandths of a milligram of iron per cc. was found to produce a considerable acceleration.

A number of experiments were then carried out using purified thioglycollic acid instead of cysteine and it was found that in marked contrast to cysteine, the rate of reduction of methylene blue by purified thioglycollic acid was not appreciably increased by the addition of iron in quantities of the same order as those employed in the experiments with cysteine. On the other hand, a trace of copper was found to act as a very powerful catalyst in the anaerobic oxidation of purified thioglycollic acid. This is shown in Table V.

Table V.

Copper added as CuSO_4 . Temp. 25°.				
No.	Thioglycollic acid (mg.)	p_H	Cu added (mg.)	Reduction time of Mb in min.
1	1.2	7.4	—	137
	"	"	0.001	< $\frac{1}{2}$
	"	"	0.0005	10
2	1.6	7.6	—	35
	"	"	0.001	3
3	1.2	7.9	—	155
	"	"	0.001	6

The addition of $M/1000$ HCN to the purified thioglycollic acid was found to produce practically no inhibition, though the oxidation of a solution of the pure thioglycollic acid to which a minute trace (0.0005 mg.) of copper had been added was markedly inhibited by $M/1000$ HCN.

In investigating the effect of iron on the anaerobic oxidation of purified thioglycollic acid, a slight acceleration appeared to be induced by relatively large quantities of iron, but in view of the extremely powerful effect of copper on this oxidation, it seems possible that this effect observed with larger con-

centrations of iron may be due to traces of copper (or possibly some other catalytic metal) present in the iron solutions. Owing to the non-sensitivity of thioglycollic acid to iron, and to its ready purification by a single distillation in quartz, it is easy to obtain solutions of this compound the rate of oxidation of which is not appreciably inhibited by the addition of HCN. On the other hand, such solutions of cysteine are more difficult to obtain.

There seems little reason to believe that the action of catalytic metals on the anaerobic oxidation of pure glutathione would be essentially different from their action on pure cysteine, for in the experiments on the aerobic oxidation of purified glutathione, it was shown that traces of iron produced a similar catalytic effect on the purified glutathione to that produced on purified cysteine [Harrison, 1924]. Further, it is shown in the present paper that the anaerobic oxidation of glutathione is inhibited by traces of cyanide in a similar way to that of cysteine and thioglycollic acid. It seems highly probable, therefore, that the anaerobic oxidation of pure glutathione is catalysed by traces of metals in a similar way to that of cysteine.

DISCUSSION.

In a recent paper by Moureu, Dufraisse and Badoche [1926], it is suggested that the inhibitions caused by hydrogen cyanide are not necessarily to be explained by the inactivation by cyanide of traces of catalytic impurities, but that the facts can equally well be explained by the general "antioxygen" theory of Moureu and Dufraisse [1926]. According to this theory, the substance which is oxidising and the substance which is causing inhibition both take up oxygen to form compounds of the peroxide type. The interaction and mutual decomposition of the two peroxides then takes place. This leads to the regeneration of the original unoxidised substances, and thus oxidation is prevented. These authors suggest that the inhibition caused by hydrogen cyanide in the oxidation of sulphydryl compounds can be explained in the above manner, but that such is not the case is evident from the following considerations. Firstly, the more carefully the sulphydryl compounds are purified from traces of catalytic metals, the less becomes the inhibition produced by cyanide [Harrison, 1924]. Secondly, pyrophosphates have been shown to inhibit the oxidation of sulphydryl compounds in a similar way to cyanides, and in this case the formation of a transitory peroxide of the inhibitor (as required by the antioxygen theory) can hardly be taking place. Finally, the experiments described in the present paper show that the inhibition by cyanide also occurs in the *anaerobic* oxidation of sulphydryl compounds, a reaction in which formation of peroxides of the substance oxidising or of the inhibitor can scarcely be regarded as possible.

From the results of the experiments given so far in this paper, it is evident that the anaerobic oxidation of the sulphydryl group is accelerated by traces of iron in a similar way to its aerobic oxidation. In the experiments on the anaerobic oxidation, methylene blue replaces oxygen as the hydrogen acceptor.

Since iron produces a similar catalytic effect on both the aerobic and the anaerobic oxidations of the sulphhydryl group, it would appear that the iron cannot be acting as an activator of oxygen as suggested in Warburg's theory. It would seem rather that the experimental facts can be satisfactorily explained by assuming that in both the aerobic and anaerobic oxidation of the sulphhydryl compounds, the iron (or other catalytic metal) is acting as an intermediary catalyst, becoming alternately oxidised and reduced. Ferric iron is reduced by the sulphhydryl compound; the ferrous compound thus formed then reduces the methylene blue to methylene white in the anaerobic experiments, or oxygen to hydrogen peroxide in the aerobic experiments. The resulting ferric compound is then again reduced by a further quantity of the sulphhydryl compound, so bringing about the oxidation of the latter. The essentials of the process may be expressed as follows:

Reaction (2) will proceed in a forward direction in the presence of a hydrogen acceptor such as oxygen or methylene blue, so long as the reduction potential of the system expressed by $\text{Fe}^{\cdot\cdot}/\text{Fe}^{\cdot\cdot\cdot}$ is sufficiently high to reduce the hydrogen acceptor.

This condition is ensured by the re-reduction of the ferric iron by the excess of sulphhydryl compound in the solution, the reduction potential of the system $\text{Fe}^{\cdot\cdot}/\text{Fe}^{\cdot\cdot\cdot}$ thus being maintained. Dixon [1927] has recently described experiments which indicate that the reduction potential of the sulphhydryl system is considerably higher than that indicated by previous work. The sulphhydryl compounds will tend to maintain the reduction potential of the ferrous-ferric system at a value well above that of the system MbH_2/Mb , and the reduction of the methylene blue will proceed virtually to completion. In such a mechanism, the velocity of oxidation of the sulphhydryl compound will be determined by the slower of the two reactions, namely, the reduction of the ferric compound by the sulphhydryl group, and the subsequent reduction of the hydrogen acceptor by the resulting ferrous compound. A positive catalysis by iron will occur provided that the slower of these two reactions is more rapid than the direct reduction (without the intervention of iron) of the hydrogen acceptor by the sulphhydryl compound.

Such a conception of iron as an intermediary catalyst does not appear to necessitate the assumption of an activation of oxygen by iron as pictured by Warburg. It would appear rather that molecular oxygen is acting as an acceptor for hydrogen formed in reaction (2). It is probable that the catalysis proceeds by the alternate oxidation and reduction of an iron-sulphhydryl complex rather than by the free inorganic iron indicated in reactions (1) and (2). There is abundant evidence for the existence of this complex. The

assumption of its formation, while it does not modify in any essential the theory outlined above, will explain the non-precipitation of ferric hydroxide from the reacting system during the catalysis in alkaline solution. There seems no reason for assuming an activation of oxygen by iron in the aerobic system any more than we assume an activation of methylene blue by iron in the anaerobic system. The experimental results given in the present paper combined with previous work on the subject indicate that there is no essential difference between the catalysis in the aerobic and in the anaerobic systems, and the mechanism outlined above would explain the catalysis in the two systems on exactly similar lines. A somewhat similar explanation of the catalysis by iron of the oxidation of hexoses in phosphate solution has recently been suggested by Blix [1927].

An alternative possible explanation of the catalysis of the oxidation of sulphydryl compounds is one similar to that suggested by Toda [1926], namely, the activation of the hydrogen of the sulphydryl group by iron. In the formation of the complex between iron and the sulphydryl compounds, the hydrogen of the sulphydryl group is assumed to be activated, and this hydrogen is then readily taken up by methylene blue or oxygen. It would seem, however, that the theory of the alternate oxidation and reduction of the catalytic metal is the more attractive explanation of the facts for several reasons. Firstly, it does not involve the assumption (for which as yet there is no direct evidence) of an activation by iron of the hydrogen of the sulphydryl group. Secondly, there is definite evidence that an alternate oxidation and reduction of iron takes place during the catalytic oxidation of the sulphydryl group. The change of ferrous to ferric iron and *vice versa* can actually be observed by the appearance and disappearance of the violet colour of the ferric-cysteine complex in the alkaline oxidising solution [Harris, 1922], and it was shown by Harrison [1924] that a similar colour change occurs also in glutathione solutions oxidising in the presence of iron. Further, it appears significant in this connection that the metals which were found by Mathews and Walker [1909] to catalyse the oxidation of cysteine are all capable of existing in at least two well-defined different states of valency. The evidence given in favour of the oxidation-reduction theory of catalysis by iron does not, however, exclude the possibility that the direct activation of the hydrogen of the sulphydryl group by iron may also be playing a part. The two mechanisms may be going on side by side.

The experiments described in the present paper, while showing the enormous catalytic effect of certain metals, at the same time appear to indicate that the reduction of methylene blue by sulphydryl compounds can take place with a not inconsiderable velocity, even in the absence of catalytic metals. In the purest solutions of methylene blue and sulphydryl compounds obtained, it was found that the addition of cyanide produced no appreciable inhibition in the rate of anaerobic oxidation. Any traces of catalytic metals remaining in the purified solutions would presumably be inactivated by the

cyanide, and yet, even in the presence of cyanide, the velocity of the reaction was found to be practically unaltered. It is of interest to compare this result with the results of experiments obtained by the author [Harrison, 1924] in the case of the aerobic oxidation of glutathione and cysteine. It was found in these aerobic experiments that cyanide did indeed produce an inhibition in the rate of oxidation of the purified compounds, though, even in the presence of excess of cyanide, an appreciable oxidation of the sulphydryl compounds still occurred. It seems not unlikely, therefore, that the sulphydryl group can oxidise to some extent, even in the absence of catalytic metals, and that these metals act by accelerating a reaction which is already taking place at a measurable rate.

OXIDATION OF IRON-FREE METHYLENE WHITE.

According to Warburg, molecular oxygen can under no circumstances act as an acceptor for hydrogen; the molecular oxygen must first be activated by combination with iron. If this view be correct, it follows that the aerobic oxidation of any substance by loss of hydrogen cannot take place in the complete absence of iron. However, during the preparation of iron-free methylene blue (as described above), it was interesting to find that the iron-free leuco-compound oxidised rapidly on exposure to the air. Here was an example of a substance containing two labile hydrogen atoms which could be taken up by the molecular oxygen of the air, even in the absence of iron. The oxidation was evidently not brought about by traces of iron introduced from the air during the drying of the leuco-compound on the pump, for small quantities of leuco-compound which remained behind in the quartz flask were similarly oxidised.

Further evidence for the ability of molecular oxygen to act as a hydrogen acceptor for the labile hydrogen of leuco-methylene blue is afforded by the following experiments.

To each of two vacuum tubes were added 0.1 cc. of cysteine solution (containing 0.3 mg. of free cysteine), 0.3 cc. of 1/5000 methylene blue and 1.5 cc. of p_H 7.6 phosphate buffer. 0.1 cc. of $M/20$ KCN (previously neutralised with HCl) was then added to one tube and an equal volume of water to the other tube. The tubes were then evacuated and immersed in a water-bath at 37° until the methylene blue was completely decolorised in both tubes. The tubes were then opened to the air and shaken. It was found that the solutions in both tubes became slightly blue almost immediately, and the rate of progressive deepening was the same in both tubes. The tube to which cyanide had been added smelled strongly of HCN at the end of the experiment. In another experiment, 10 cc. of a 1/5000 solution of iron-free methylene blue in pure water was reduced by passing a current of pure hydrogen sulphide (prepared from Sb_2S_3) through the solution for half an hour. The solution was then divided into two equal parts in two quartz test tubes. To one tube was added 0.1 cc. of $M/20$ KCN (carefully neutralised with HCl) and to the

other was added an equal volume of pure water. The tubes were then evacuated on the pump to remove excess of hydrogen sulphide, and, on opening to the air and shaking, the rate of oxidation of the leuco-methylene blue was again observed to be the same in both tubes. Traces of iron impurities in the solution would have been inactivated by the hydrogen cyanide, and since the rate of oxidation was the same, either in the presence or in the absence of cyanide, it is evident that iron is not necessary for the oxidation of leuco-methylene blue, but that the latter can give up hydrogen directly to unactivated molecular oxygen.

This fact is of importance in considering the validity of Warburg's theory. Warburg states that molecular oxygen is entirely unable to act as an acceptor for hydrogen unless first activated by iron. Nevertheless, the above experiment proves that molecular oxygen is capable of combining with the hydrogen of leuco-methylene blue, even in the complete absence of iron. It would appear, therefore, that in certain oxidations at any rate, molecular oxygen *can* function directly as a hydrogen acceptor. It is true that the oxidation of leuco-methylene blue is not actually a biological reaction, but there seems no reason why it should be regarded as essentially different in mechanism from the oxidation of a biological system. As shown by Dixon and Thurlow [1925], the aerobic oxidation of hypoxanthine in the presence of the xanthine oxidase is also unaffected by the addition of cyanide. It would therefore seem that both in the oxidation of leuco-methylene blue and in the oxidation of hypoxanthine in the presence of xanthine oxidase, labile hydrogen is taken up by molecular oxygen without the intervention of iron. Warburg [1924], however, refused to concede that the oxidation of hypoxanthine in the presence of xanthine oxidase is a tissue respiration system. It is difficult to understand why this reaction should not be classed as a part of tissue respiration, since the oxidation of hypoxanthine is known to occur in the tissues.

SUMMARY AND CONCLUSIONS.

Briefly, the chief conclusions that may be drawn from this paper are as follows. The fact that hydrogen cyanide inhibits not only the aerobic but also the anaerobic oxidation of sulphydryl compounds indicates that it is not inhibiting an oxygen activator. Such a fact means that inhibition by cyanide in an oxidising system does not justify the assumption that oxygen activation is a necessary process in that system. The observation that the addition of very small traces of certain metals to purified sulphydryl compounds accelerates not only the aerobic but also the anaerobic oxidation of the sulphydryl group affords further indication that the catalytic action of these metals is not that of an oxygen activator. It is suggested that this catalysis of the oxidation of the sulphydryl group by metals is effected by the alternate oxidation and reduction of the catalytic metal by means of which hydrogen is made available for acceptance by molecular oxygen or by methylene blue. Such an explanation makes comparable the mechanism involved in the catalysis of both the

aerobic and the anaerobic oxidation of the sulphydryl compound. These experiments indicate the important part played by extremely minute traces of iron and other metals in biological oxidations involving the sulphydryl group. Copper, in the concentrations shown to be effective, may well be regarded as a physiological factor, since small quantities of copper have been shown to be present in many living tissues. Another possible function of small quantities of iron has been indicated by Harrison and Thurlow [1926] in whose experiments it was shown that in the presence of the xanthine oxidase system to which ferrous iron had been added, the oxidation of certain physiological substances such as lactic acid and β -hydroxybutyric acid could be brought about. In this system, the iron appears to be acting as a peroxidase rather than by the mechanism considered above.

The experiments in the present paper indicate that the reduction of methylene blue by sulphydryl compounds may take place to an appreciable extent even in the complete absence of catalytic metals. A similar result was obtained in an earlier paper in the aerobic oxidation of sulphydryl compounds. Further evidence against the necessity for oxygen activation in this system is thus afforded. The fact that iron-free leuco-methylene blue has been shown to be strictly autoxidisable affords another example of the direct acceptance of hydrogen by molecular oxygen.

The author wishes to express his sincere thanks to Sir F. G. Hopkins and to Professor E. Mellanby for their interest and encouragement.

He also wishes to express his indebtedness for a Keddey Fletcher-Warr Studentship, University of London, held during the course of this work.

REFERENCES.

- Blix (1927). *Skand. Arch. Physiol.* **50**, 8.
Dixon (1927). *Proc. Roy. Soc. Lond. B*, **101**, 57.
Dixon and Thurlow (1925). *Biochem. J.* **19**, 672.
Dixon and Tunnicliffe (1923). *Proc. Roy. Soc. Lond. B*, **94**, 281.
Harris (1922). *Biochem. J.* **16**, 739.
Harrison (1924). *Biochem. J.* **18**, 1009.
Harrison and Thurlow (1926). *Biochem. J.* **20**, 217.
Mathews and Walker (1909). *J. Biol. Chem.* **6**, 299.
Moureu and Dufraisse (1926). *Chem. Rev.* **3**, 113.
Moureu, Dufraisse and Badoche (1926). *Compt. Rend. Acad. Sci.* **183**, 685.
Sakuma (1923). *Biochem. Z.* **142**, 68.
Thurlow (1925). *Biochem. J.* **19**, 186.
Toda (1926). *Biochem. Z.* **172**, 34.
Warburg (1924). *Biochem. Z.* **152**, 479.