CXCIV. THE DEHYDROGENASES OF *BACTERIUM COLI*.

II. THE RATE OF REDUCTION OF METHYLENE BLUE.

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In a previous paper of this series [Yudkin, 1933] it was shown that the reduction time of a quantity of methylene blue by a washed suspension of *Bact. coli* in the presence of a substrate depended on the interaction of several factors. These were:

1. The temperature lag effect, that is, the slower rate of reduction of the dye at the beginning of the experiment while the reactants are acquiring the temperature of the bath.
2. The presence of residual oxygen in the Thunberg tube.
3. The gradual poisoning of the enzyme by methylene blue.
4. The affinity of the enzyme for methylene blue.
5. The presence of hydrogen donors in the bacterial suspension.
6. The products of the action of the bacteria on the substrate which can act as further hydrogen donors.

The first four of these will tend to increase the reduction time, the last two to decrease it.

In this paper, the study of the rate of reduction of methylene blue was undertaken with a view to demonstrating more clearly the part played by each of these factors in determining the reduction time.

The rate of reduction of methylene blue is always held to be linear for the greater part of the reaction. In the case of the succinic dehydrogenase of *Bact. coli*, it was shown colorimetrically that the reduction is linear until about 90 % of the dye is reduced, after which the rate falls off [Quastel and Whetham, 1924]. This falling off in the rate when the dye concentration has become small is explained by the affinity of the dye for the enzyme: that is, the concentration of the methylene blue has reached a value below that at which it is able to saturate the enzyme.

The rate of reduction of methylene blue has also been studied electro-metrically with yeast suspensions [Cannan *et al.*, 1926]. Here it is stated that the reduction is linear throughout.

EXPERIMENTAL.

The preparation of the bacterial suspension and the reduction of methylene blue in Thunberg tubes were carried out as described in the first paper of this series. The same three enzymes were investigated, namely formic, succinic and glucose dehydrogenases. The rate at which the methylene blue was reduced

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was followed colorimetrically, the gradually decreasing depth of colour in the experimental tubes being compared with a series of standard tubes containing varying quantities of methylene blue, 1 ml. of the bacterial suspension being used in the experiment and water to bring the volume to 5 ml. [Quastel and Whetham, 1924].

All experiments were done at least in duplicate. With times of 20 minutes or more, duplicate values for any given degree of reduction usually agreed within 5 %, always within 10 %. The error was larger for smaller reduction times. However, on drawing a smooth curve through the points obtained from any pair of duplicates, these errors largely cancelled out, so that two pairs of duplicates done with exactly the same bacteria *etc.* gave curves which could be very well superimposed.

RESULTS.

1. Rate of reduction of methylene blue by formic, succinic and glucose dehydrogenases.

A typical series of results is shown in Fig. 1.

Fig. 1. Rate of reduction of methylene blue.

Δ Formic dehydrogenase. • Succinic dehydrogenase. ○ Glucose dehydrogenase.

With succinic dehydrogenase is seen very well the effect of Factor 5 mentioned above—the acceleration due to hydrogen donors in the suspension. These are rapidly used up, giving a decreased rate of reduction. The linear reduction for the first 80 % and then a falling off obtained by Quastel and Whetham [1924] for the same enzyme might be due to the fact that their reduction time was much less—about 9 minutes. There would therefore be a greater error due to temperature lag and residual oxygen (Factors 1 and 2) which would act in the opposite direction to Factor 5, and would also be manifest in the earlier part of the experiment.

In the case of the other two enzymes, there is seen an initial lag in the rate of reduction, particularly with formic dehydrogenase. This is due to Factors 1 and 2. The lower magnitude of this effect in the case of glucose dehydrogenase, and its complete absence in the case of the succinic enzyme, may be explained by the fact that the suspensions of bacteria used for these two enzymes were much stronger. For the glucose enzyme it was about 10 times, and for the succinic

enzyme about 50 times more concentrated than for the formic enzyme. There were therefore more hydrogen donors in these suspensions which partially or completely masked the lag effects of temperature and oxygen.

The decreasing rate of reduction towards the end, due to the affinity of the enzymes for the dyestuff, is well shown for each of the three dehydrogenases.

2. *Effect of dilution on the rate of reduction.*

The rate of reduction with succinic dehydrogenase was followed with two suspensions of bacteria, one being one-third as concentrated as the other (Fig. 2).

Fig. 2. Rate of reduction of methylene blue: effect of dilution. Succinic dehydrogenase.

● Undiluted. ■ Diluted 1 in 3.

The scale of the abscissa for the curve with the diluted suspension is three times that of the more concentrated, so that the effects of dilution are shown more clearly.

The initial relatively more rapid reduction with the more dilute suspension may be accounted for by the relatively smaller lag effect here, where the actual time for reduction is greater. The rate with the diluted suspension then begins to fall off due to the poisoning with methylene blue (Factor 3). Towards the end of the experiment, however, the rate increases again before the final falling off. This seems to be due to an activation of the enzyme during this period at 40°. An indication of this phenomenon was obtained in the previous work, in which a slightly more rapid reduction occurred with succinic acid after the bacteria had been warmed for one hour at 40°, compared with bacteria kept at 0°. This is not due to a liberation of donors from the bacteria themselves since there is no corresponding decrease in reduction time in the controls without succinate after the bacteria had been kept at 40° for one hour.

The figure shows that the effect of dilution on the reduction time depends on the end-point chosen. If 80 % reduction is taken, the reduction time of a suspension diluted three times is rather more than three times that of the undiluted (85 minutes and 25 minutes). If complete reduction is taken, then the diluted suspension gives a reduction time rather less than three times that of the undiluted (112 minutes and 38 minutes).

The curves for glucose (Fig. 3) show not only the factors mentioned above but also the effect due to the coenzyme [Yudkin, 1933]. Although the bacteria in the diluted suspension were two-thirds as concentrated as in the undiluted, the scale of the abscissa for the results with the former had to be three times that of the latter in order to bring them together as shown.

Fig. 3. Rate of reduction of methylene blue: effect of dilution. Glucose dehydrogenase.

• Undiluted. □ Diluted 1 in 3.

3. Reduction of varying quantities of methylene blue.

(a) *Times for complete reduction.* In order to avoid the prolonged action of methylene blue on the enzymes in experiments where the latter were unavoidably dilute, attempts were made to use smaller quantities of the dyestuff. The results obtained on taking the times for complete reduction of different quantities of methylene blue show very striking differences for the different enzymes. Succinic dehydrogenase shows a decreasing reduction time for decreasing amounts of methylene blue, whilst with formic acid, when the concentration of dye is below a certain value, the times taken to reduce very different quantities of the dye are approximately the same (Fig. 4). The results for glucose dehydrogenase are similar to those for formic dehydrogenase (Fig. 5).

These somewhat extraordinary results were found to be readily explicable by a consideration of the factors detailed above, which are involved in determining the time of reduction. These factors are well brought out by a study of the rates of reduction of these varying quantities of methylene blue.

With succinic dehydrogenase (Fig. 6), the rapid reduction of the small quantities of methylene blue is seen to be due in the main to the reduction of the dye by the other donors present (Factor 5). With higher dye concentration is seen the acceleration in the latter half of the reduction which we have suggested is due to an activation of the enzyme.

With the other two enzymes (Figs. 7 and 8) it is clear that the relatively long reduction time for the smaller quantities of dye can be explained by Factors 1-3. The first two of these factors—the lag due to residual air and temperature—will almost certainly have disappeared after the first 10 minutes. Yet it will be seen that the slow final rates of reduction with smaller quantities

of dye are much slower than the final rates of corresponding quantities of dye in those experiments where the initial concentration of dye was higher. Thus, when 1 ml. of methylene blue was used, the part of the curve showing the rate of reduction of the last 0.2 ml. of methylene blue is much steeper than the

Fig. 4. Time for complete reduction of varying quantities of methylene blue.
 Δ Formic dehydrogenase. $\bullet$ Succinic dehydrogenase.

Fig. 5. Time for complete reduction of varying quantities of methylene blue. Glucose dehydrogenase.

corresponding part of the curve for the rate of reduction of 0.25 ml. These results may be explained by an accumulation of secondary products of the reaction between bacteria and substrate (Factor 6). This explanation was tested by experiments in which, after a certain quantity of dye had been reduced, a second quantity was added.

(b) *The effect of addition of a second quantity of methylene blue.* For these experiments, Thunberg tubes were used in which the second quantity of dye was contained in a side-arm. This was mixed with the other reactants immediately after the reduction of the first quantity of dye.

Fig. 6. Rate of reduction of varying amounts of methylene blue. Succinic dehydrogenase.

Fig. 7. Rate of reduction of varying amounts of methylene blue. Formic dehydrogenase.

Fig. 8. Rate of reduction of varying amounts of methylene blue. Glucose dehydrogenase.

Formic and glucose dehydrogenases reduce the second quantity of methylene blue much more rapidly than the first, whilst succinic dehydrogenase does the reverse (Table I).

Table I.

Dehydrogenase	Reduction time of 0.5 ml. M.B. in mins.		Reduction time of 0.25 ml. M.B. in mins.	
	1st 0.5 ml.	2nd 0.5 ml.	1st 0.25 ml.	2nd 0.25 ml.
Formic	26	13	25.5	9.5
Glucose	16.5	12	15.5	8
Succinic	43	73	20	30

Fig. 9. Rate of reduction of methylene blue. Formic dehydrogenase.

Fig. 10. Rate of reduction of methylene blue. Formic dehydrogenase.

The results with the first two enzymes might be due in part to the lag effects which, of course, manifest themselves only during the first half of each experiment. However, on following the rate of reduction it is seen that there is a real increased rate of reduction; the reduction now proceeds as if the whole amount of the dye had been present originally (Figs. 9 and 10). The curves after the

addition of the second amounts of dye can be well superimposed on the lower part of the curves showing the reduction of the double quantity of methylene blue. Exactly similar results were obtained with glucose dehydrogenase.

A more rapid reduction of a second quantity of methylene blue has also been shown for yeast [Ambrus *et al.*, 1931]. Here, however, it was shown to be due not to an increase in the quantity of hydrogen donors but to an increased penetration of the dye into the organisms.

The fact that succinic dehydrogenase gives a greater reduction time for the second quantity of methylene blue is to be expected from Fig. 6.

4. Effect of addition of leucomethylene blue.

It has been shown that an accelerated reduction occurs with the enzymes which is due to one or more of the products of reaction. It has hitherto been assumed that these are the products of the action of the bacteria on the substrate. However, the possibility existed that the other product of the reaction, namely leucomethylene blue, might produce this effect.

This hypothesis was tested in the following way (Fig. 11). Except for the rubber stoppers in the three boiling-tubes, the apparatus consisted entirely of glass. Methylene blue was reduced by a palladium catalyst, prepared by the precipitation of palladium on to barium sulphate by reduction with formaldehyde. Some dye, together with about 20 mg. of catalyst, was placed in each of tubes 1 and 2, and hydrogen passed until reduction was effected. This took about 10 minutes. Nitrogen, freed from oxygen by passing over palladium-copper heated to 380°, was passed for $\frac{1}{2}$ – $\frac{3}{4}$ hour to remove the excess of hydrogen. Tube 1, with leucomethylene blue, served to indicate whether the nitrogen was in reality oxygen-free. Actually, no coloration took place after running the purified nitrogen for several hours. Leucomethylene blue was now drawn up into the burette, which had previously been freed of air by passing nitrogen.

Fig. 11.

Two tubes, each containing equal quantities of buffer solution (p_H 7.0) and substrate ($M/10$) were cooled to 0°, and then equal volumes of cooled bacterial suspension were added to each. To one were added 2 ml. of distilled water (3). The other replaced tube 2. Both were kept in ice whilst nitrogen was passed for $\frac{1}{2}$ – $\frac{3}{4}$ hour to remove air. Then into tube 2 were run 2 ml. of leucomethylene blue from the burette, the ice was replaced by water at 40°, and the times for complete reduction in the two tubes were observed.

It will be seen (Table II) that the addition of the leuco-dye has no effect on the reduction. The acceleration noted above must therefore be due to the interaction of bacteria and substrate.

Table II.

Dehydrogenase	Reduction time, mins.	
	Without leucomethylene blue	With leucomethylene blue
Formic	35	36
Glucose	27	28
Succinic	55	54

DISCUSSION.

It has been seen that there is an accelerated reduction due to the formation of products from the substrate which can act as further hydrogen donors. What these products are in the case of formic acid it is difficult to know. One possibility would be the production of molecular hydrogen, which is known to act as a hydrogen donor with *Bact. coli* [Stephenson and Stickland, 1931]. But *Bact. coli*, grown as in this work in Roux bottles on caseinogen broth, is unable to liberate measurable quantities of hydrogen from formate [Yudkin, 1932]. It has also been shown that, in disagreement with the results with glucose and succinate, the effect of leaving bacteria in the presence of formate does not give a definite acceleration. There seem to be two possibilities. The first is that very small quantities of hydrogen are indeed produced, sufficient to reduce part of the methylene blue, but not sufficient to be demonstrated in the technique used for determining the amount of hydrogenlyase [Yudkin, 1932]. The second possibility is that in the presence of methylene blue products are formed from the formate which are different from those formed in its absence. Again, what such products are is difficult to imagine.

SUMMARY.

The several factors involved in determining the "reduction time" of methylene blue are well brought out by a study of the rate of reduction of the dye. Other phenomena, such as the difference in behaviour of formic dehydrogenase and succinic dehydrogenase in reducing varying quantities of methylene blue are also readily explained by these factors as shown by following the rate of reduction.

It has been shown that the presence of leucomethylene blue does not influence the reduction time of the dehydrogenases of glucose, formic acid or succinic acid.

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