CCLII. THE DEHYDROGENASES OF *BACTERIUM COLI*.

I. THE EFFECT OF DILUTION: WITH A NOTE ON THE EXISTENCE OF A CO-ENZYME OF GLUCOSE DEHYDROGENASE.

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THE activity of an enzyme preparation is normally proportional to the amount of enzyme present. Quastel and Whetham [1924] showed that this proportionality existed in the case of the succinic dehydrogenase of *Bact. coli*. Although this was the only dehydrogenase studied, and although the dilution was made only over a small range, it has since been assumed that such proportionality holds for all the dehydrogenases, and hence, that over a wide range of reduction times the reciprocal of these times is a true measure of the amount of the enzyme under investigation.

During a study of the effect of poisons on the dehydrogenase systems of *Bact. coli*, it soon became evident that such assumptions were not entirely justifiable. The effect of dilution has, therefore, been investigated for three dehydrogenases of this bacterium. The results, recorded below, led to a detailed investigation of each of the several factors involved in the methylene blue technique of Thunberg, as developed by Quastel for the study of the bacterial dehydrogenases. As usually followed, this consists in observing the time of reduction of a given quantity of methylene blue *in vacuo* in the presence of a suspension of washed bacteria by a given substrate at a definite temperature in the presence of a buffer solution.

EXPERIMENTAL.

A washed suspension of *Bact. coli* was used. The bacteria were grown in Roux bottles for 15–18 hours on a tryptic digest of caseinogen. They were centrifuged, washed twice by centrifuging in distilled water and finally made up in uniform suspension in distilled water. Such a preparation showed no appreciable differences in the properties of the dehydrogenases here investigated from one in which the bacteria were suspended in Ringer solution instead of distilled water. The enzymes studied were formic acid, succinic acid and glucose dehydrogenases. The normal procedure, which in several experiments has been modified as described later, was as follows. 1 cc. each of distilled water, phosphate buffer p_H 7.0 ($M/20$), methylene blue (1/5000), substrate solution and bacterial suspension were placed in a Thunberg tube. The final volume was always 5 cc. The tube was evacuated for about $1\frac{1}{2}$ minutes at the water-pump, then placed in a bath at 40°. This was taken as the beginning of the experiment

and the time taken for the methylene blue to become reduced by 80 % as estimated by comparison with standards was taken as the reduction time. Each experiment was done at least in duplicate. The substrates were *M*/10 solutions of sodium formate, sodium succinate and glucose. The bacterial suspension used was of different concentrations for the different enzymes, being strongest for the succinic and most dilute for the formic dehydrogenase. Thus similar reduction times were obtained for the three dehydrogenases.

Effect of dilution.

If a strict proportionality exists between enzymic activity and dilution, then the product of the dilution and the activity should be constant for all degrees of dilution. The activity of the dehydrogenases may be taken as the reciprocal of the reduction time. If now the product of activity and dilution is plotted against the reduction time, the limits of reduction time between which proportionality holds can be read off directly. In Fig. 1 the results for the three

Fig. 1. Effect of dilution on the dehydrogenases.

A. Formic dehydrogenase, Δ . B. Succinic dehydrogenase, $\bullet$. C. Glucose dehydrogenase, $\circ$.

enzymes are so plotted. In order that the three curves might be plotted on the same figure, the maximum value of the product of activity and dilution has been given an arbitrary value of 100 units.

Similar curves are of course obtained on plotting the product of activity and dilution against dilution.

It will be seen that formic dehydrogenase exhibits strict proportionality between dilution and activity when the reduction time lies between 20 and 160 minutes. After this, the activity begins to decrease rather more rapidly than is warranted by the dilution. Succinic dehydrogenase shows this proportionality between reduction times of 20 and 50 minutes. With both enzymes, when the reduction time is less than 20 minutes the activity is too low, *i.e.* the reduction time is too long, a result mainly due to the errors introduced by the relatively large temperature coefficient of the reaction effecting an increasing rate until the materials attain the temperature of the bath¹.

Another possible source of error is the reduction of some of the methylene blue before the tube is placed in the bath. The results show clearly, however, that this effect, which would tend to give too low a reduction time, is completely masked by the temperature lag effect.

¹ The presence of small quantities of residual air would have a similar effect.

The results with glucose dehydrogenase are very striking. There is no range at all within which there is any proportionality between activity and dilution. When the reduction time is less than 10 minutes, a rise in the curve occurs as with the other two enzymes. In this case, however, the rise does not continue until a reduction time of 20 minutes is reached and then remain constant, but a rapid fall in the curve occurs when the reduction time exceeds 10 minutes. The effect of factors responsible for the extreme lack of proportionality with glucose dehydrogenase would seem to exceed the temperature lag effect when the reduction time is more than 10 minutes.

The lack of proportionality at the higher reduction times might be due to a gradual destruction of the enzymes at 40° or to a slow poisoning of the enzymes by methylene blue. The phosphate buffer and the substrate itself might also be influencing the reduction times. All these factors have, therefore, been investigated for each of these three enzymes.

Inactivation of the enzymes at 40°. It is well known that on keeping a suspension of *Bact. coli* for some time, even at 0°, there is a decrease in the activity of the enzymes. By far the most susceptible of the three here studied is the glucose dehydrogenase. Quastel and Wooldridge [1927] showed that on keeping a suspension of *Bact. coli* for one hour at higher temperatures (47° and 57°) formic dehydrogenase was unaffected, succinic affected appreciably at 57°, whilst the glucose dehydrogenase was appreciably affected at 47° and completely inactivated at 57°.

It therefore seemed likely that glucose dehydrogenase was being appreciably inactivated at the temperature of the bath (40°) and that this might in part account for the decreased activity noted when the concentration of the bacteria was such as to give a fairly long reduction time.

Suspensions of the bacteria were kept at 40° for one hour and the activities compared with suspensions kept at 0° (Table I). It will be seen that this treatment causes no diminution in the activity of any of the enzymes.

Table I.

Dehydrogenase	Reduction time in minutes after one hour at	
	0°	40°
Formic	17.75	18.25
Succinic	22.5	20.0
Glucose	10.75	10.25

Poisoning by methylene blue. 1 cc. of methylene blue solution was placed in each of a number of Thunberg tubes and 1 cc. of bacterial suspension added. At varying intervals, 1 cc. each of buffer solution, substrate solution and water were added to these tubes, which were then evacuated and the reduction times taken.

The results are given in Fig. 2 in which the original activity of the enzyme has been given a value of 100 units. Formic dehydrogenase is entirely unaffected by methylene blue in 5 hours. Succinic dehydrogenase is poisoned to the extent of 40 % after 2 or 3 hours. Glucose dehydrogenase is poisoned to the extent of 80 %; at first the poisoning is rapid, then it becomes much slower.

The poisoning action of methylene blue in these experiments took place at 19–20°. An experiment with glucose dehydrogenase at 40° showed that the poisoning was much more rapid but the final activity of the poisoned enzyme was the same. Glucose dehydrogenase is also poisoned to the same extent ~~in vacuo~~.

In these experiments, the poisoning action of methylene blue continues during the period of reduction. The times given on the abscissa in Fig. 2 are the times from the addition of the methylene blue to the bacteria to the time when put in the bath with the other substances. These times are, therefore, less than the actual time of action of the methylene blue.

Fig. 2. Poisoning of the dehydrogenases by methylene blue.

A. Formic dehydrogenase at 19°, Δ . B. Succinic dehydrogenase at 19°, $\bullet$.
 C. Glucose dehydrogenase at 19°, $\circ$. D. Glucose dehydrogenase at 40°, $\oplus$.

Since dilution involves more prolonged action of the dye on the enzyme, the poisoning effect of methylene blue seems at first glance to account for the observed discrepancies in the dilution experiments, since the enzyme most affected by dilution is the one most affected by methylene blue. Formic dehydrogenase, which is little influenced even by great dilution, is unaffected also by methylene blue. The poisoning experiments might also account completely for the falling off in the activity of succinic dehydrogenase when the reduction time is greater than 50 minutes, since even at 19° an appreciable amount of poisoning has taken place in this time. In the case of glucose dehydrogenase, however, even though the enzyme has been poisoned to the extent of 50 % in one hour at 40°, this would hardly account for such a great fall in the activity as that observed when the reduction time is increased from 10 minutes to, say, 25 minutes. It is probable, therefore, that there are other factors which cause this phenomenon in the case of glucose dehydrogenase.

Effect of phosphate buffer. The effect of leaving the bacteria in contact with the phosphate buffer was investigated in a manner exactly analogous to that described for methylene blue. The suspension of bacteria was left in contact

Table II.

Dehydrogenase	Time of action of phosphate on bacteria minutes	Reduction time minutes
Formic	0	18.5
	15	18.5
	35	19.5
Succinic	0	36.0
	120	24.0
Glucose	0	15.5
	40	13.5
	60	11.5
	120	10.5

with the phosphate at room temperature, the other substances added after varying intervals and the reduction time determined (Table II).

Phosphate has very little effect on formic dehydrogenase; if anything, it results in a slight inhibition of the enzyme. The actions of both the succinic and glucose dehydrogenases are accelerated by leaving the bacteria in the presence of the phosphate for a time. Maximum acceleration, about 30 %, is obtained after about 2 hours at a temperature of about 20°.

The activation of succinic and glucose dehydrogenases by phosphate is readily reversed by removal of the phosphate by centrifuging. The activities of the enzymes determined in the ordinary way are found to be the same as before treatment with phosphate (Table III).

Table III.

Dehydrogenase	Reduction time in minutes		
	Initially	After action of phosphate for 2 hours	After removal of phosphate
Succinic	31.0	21	33
Glucose	11.25	8	12

Effect of phosphate buffer on the poisoning by methylene blue. The bacterial suspension was left in contact with both phosphate buffer and methylene blue for an hour and the activities of the enzymes determined after the addition of the substrates.

Table IV.

Dehydrogenase	Reduction time in minutes		
	Initially	After action of methylene blue for one hour	After action of methylene blue and phosphate for one hour
Formic	13.0	13.25	13.25
Succinic	17.5	21.25	21.0
Glucose	8.0	15.5	15.25

It will be evident from Table IV that the presence of phosphate has no effect on the poisoning by methylene blue of any of the enzymes. That is, the accelerating effect of phosphate is not observed in the presence of methylene blue.

Effect of using buffers other than phosphate. Although the effect of phosphates is masked by the presence of methylene blue, it was thought that an acceleration might occur in the ordinary procedure adopted in the methylene blue technique. In an attempt to avoid this possible effect of phosphate, two other buffers were tried. Sodium maleate buffer was prepared according to the description of Temple [1929]. Although Temple describes it as a buffer only for the range p_H 5.2–6.8, it can readily be shown that even at p_H 7.0, it still has about 10 % of its maximum buffering power. It was accordingly prepared and used at p_H 7.0. The second buffer prepared was the diethyl barbiturate (veronal) buffer of Michaelis [1930].

These buffers were used in a concentration of $M/20$ and compared with phosphate buffer. The activities of the enzymes were determined in the ordinary way. In certain experiments, 1 cc. of phosphate solution was added as well as the 1 cc. of the other buffer. The results are recorded in Table V.

Table V.

Dehydrogenase	cc. of buffer used			Reduction time in minutes
	Phosphate	Veronal	Maleate	
Formic	1	0	0	18.25
	1	1	0	18.0
	0	1	0	26.0
	1	0	1	23.0
	0	0	1	25.0
Succinic	1	0	0	22.5
	1	1	0	23.0
	0	1	0	31.0
	1	0	1	27.0
	0	0	1	27.25
Glucose	1	0	0	17.0
	1	1	0	30.0
	0	1	0	28.5
	1	0	1	19.0
	0	0	1	24.0

All three dehydrogenases are inhibited by veronal to the extent of 30–50 %. Glucose dehydrogenase is inhibited by veronal to about the same extent in the presence or absence of phosphate; the other two enzymes, however, have the same activity in the presence of veronal and phosphate as in phosphate alone. Maleate buffer also inhibits all three enzymes, though not equally. Glucose dehydrogenase is much less inhibited if phosphate is present at the same time; formic dehydrogenase is only slightly less inhibited, whilst succinic dehydrogenase shows the same inhibition in the presence or absence of phosphate.

Thus the three enzymes differ considerably in their behaviour towards the three buffers. The exact interpretation of these results is at present impossible, although it seems likely that in some cases phosphate plays an active part in the dehydrogenation.

Effect of acting on substrate. The suspension of bacteria was left for a time in contact with one or other of the substrates. After a time, the other substances were added and the reduction time determined.

(a) *Formic dehydrogenase.* The results with this enzyme were rather variable but in no case was there any significant alteration in the reduction time.

(b) *Succinic acid.* Bacteria left in contact with succinate show an accelerated reduction rate. It is known that succinic acid gives rise by the action of *Bact. coli* [Woelf, 1929] to malic acid, which can act as a hydrogen donator [Quastel and Whetham, 1925]. This would explain the more rapid reduction of the methylene blue after the bacteria have acted for a time on the succinate.

The acceleration due to succinate is slightly less than that due to phosphate. Phosphate and succinate together do not have an additive effect (Table VI).

Table VI.

	Minutes
Reduction time, initial	44
Reduction time, after 30 mins. at 20° with succinate	28
" " succinate and phosphate	30
" " phosphate	31

(c) *Glucose dehydrogenase.* With glucose, as with succinate, a markedly increased activity occurs after the bacteria have been in contact with the substrate for a short time before the addition of the methylene blue. In one experiment, the reduction time determined in the normal way was 13 minutes.

The reduction time after the bacteria had acted on the glucose for 15 minutes at 20° was 9 minutes. An even greater acceleration occurs if the bacteria are allowed to act on the glucose *in vacuo*.

As with succinate, these results may be explained by the formation of a substance or substances which are able to act as additional donors of hydrogen. It is well known that the action of *Bact. coli* on glucose results in the formation of several substances, such as lactic, acetic, succinic and formic acids, ethyl alcohol, etc. [Harden, 1901; Grey and Young, 1921; Aubel, 1926]. Of these, formic, succinic, lactic and acetic acids can act as hydrogen donors to methylene blue in the presence of washed suspensions of *Bact. coli* [Quastel and Whetham, 1925]. Moreover, Cook and Haldane [1931] obtained a gradually increasing rate of oxygen uptake for *Bact. coli* in the presence of glucose, which they attributed to the formation of secondary metabolites such as those considered above.

The following experiments are in accordance with the view that the acceleration is due to the production of substances from glucose which can be activated by enzymes of the bacteria to donate hydrogen to methylene blue.

Exp. 1. A suspension of bacteria was left in contact with glucose at three different temperatures. It will be seen from Table VII that no acceleration occurred when the bacteria and glucose were left at 0°, slight acceleration when they were at 20°, and a greater acceleration when they were at 40°.

Table VII.

	Minutes
Reduction time, initial	10·0
Reduction time, after 15 mins. with glucose at 0°	10·25
" " 20°	8·75
" " 40°	6·75

Exp. 2. The bacteria and glucose were left for 30 minutes at 20°, the mixture was then placed in boiling water for one minute; methylene blue was added and the tube evacuated. No reduction occurred after 6 hours.

Exp. 3. A suspension of bacteria was allowed to act on glucose for 30 minutes at 20°. 1 cc. of *N*/1000 solution of silver sulphate was added¹. Again, no reduction was observed even after 6 hours.

Exp. 4. It has been shown [Quastel and Wooldridge, 1927] that glucose dehydrogenase is completely inactivated by shaking the bacteria for 2 or 3 minutes with toluene. Now the bacteria were allowed to act on glucose for 30 minutes at 20° and were then treated with toluene; it was found that a slow reduction of methylene blue occurs in these conditions. A control experiment, in which the bacteria were treated with toluene before the addition of the glucose, showed no reduction of methylene blue whatever.

From these experiments it is obvious that the action of *Bact. coli* on glucose results in the formation of substances which are capable of being activated as hydrogen donators by enzymes present in the bacteria. Exp. 4 shows that these enzymes are not identical with glucose dehydrogenase.

The effect of glucose on the reduction of methylene blue by *Bact. coli* was similar to its effect with spores of *B. subtilis* [Tarr, 1933]. The reduction time was less after the spores had been left with glucose for 30 minutes at 40°. This was attributed by Tarr to the gradual formation of glucose dehydrogenase in the spores. It seems much more likely that here too there is an accumulation of secondary hydrogen donors, as suggested above for *Bact. coli*. This is sup-

¹ This concentration of silver sulphate has been shown to inhibit completely many dehydrogenases, such as those of glucose, lactic acid and succinic acid.

ported by the observations of the same author that spores treated with ethylurethane cannot reduce methylene blue in the presence of glucose, unless they have been allowed to act on the glucose before the addition of the ethylurethane. This is exactly similar to the results of the treatment of *Bact. coli* with toluene described above. Moreover, *B. subtilis* spores show a gradually increasing rate of oxygen uptake with glucose [Tarr, 1933], which, like the results of Cook and Haldane [1931] for *Bact. coli*, can be best explained by the formation of breakdown products of glucose.

Co-enzyme of glucose dehydrogenase.

It has been pointed out that the poisoning of glucose dehydrogenase by methylene blue is not sufficient to account for the very rapid falling off in the activity of this enzyme on dilution. Moreover, the accelerating effects of the products of the action of the organism on glucose would be expected to result in an increased rate of reduction of methylene blue when the reduction times are longer, and hence these effects would tend to compensate for the poisoning effect of the dye. It is, therefore, evident that the effect of dilution on glucose dehydrogenase is due to some other factor or factors which have not yet been considered.

It was thought possible that there existed in *Bact. coli* a co-enzyme for glucose dehydrogenase, which might be washed out of the bacteria on dilution and so become the limiting factor in the action of the enzyme. This hypothesis was tested by adding, together with untreated bacteria, 1 cc. of a suspension which had been heated to 100° for 5 minutes to the usual reactants in a Thunberg tube. The reduction time of this was taken and compared with the reduction time of glucose dehydrogenase without the addition of heated bacteria. The results are given in Table VIII. There is a considerable acceleration by the addition of heated bacteria, which themselves have no activity.

Table VIII. *Effect of addition of heated bacteria on glucose dehydrogenase.*

Bacteria	Reduction time
Unheated bacteria	33 minutes
Unheated + heated bacteria	11.75 minutes
Heated bacteria	> 6 hours

It was considered possible that the heating of the suspension might have resulted in the liberation of substances which could act as hydrogen donors and hence give an accelerated reduction of methylene blue. That this is not so is shown by the fact that a mixture of heated and unheated bacteria in the absence of glucose causes no reduction of methylene blue even after 3 hours.

The effects of dilution on glucose dehydrogenase in the presence and absence

Table IX.

Concentration of suspension	Without heated bacteria		With heated bacteria	
	Reduction time minutes	Activity × dilution	Reduction time minutes	Activity × dilution
1	8	100	8	100
2/3	11	105	12	102
1/2	17	98	17	100
1/3	83	31	22.5	112
1/5	> 360	0	35	117
1/8	> 360	0	72	92
1/12	—	—	125	80
1/16	—	—	240	56
1/24	—	—	500	40
			(approx.)	

of heated bacteria is shown in Table IX. The same undiluted heated suspension was added in each experiment.

The effect of the addition of heated bacteria is very marked in some dilutions. When the bacteria were diluted to 1/5 of their original concentration, the reduction time was too long to be measured; when the heated suspension was present, however, the reduction time was 35 minutes. It will be seen that when the concentration of bacteria is sufficient to give a reduction time of 17 minutes or less, the addition of the heated suspension is without effect. In other words, in these concentrations, there is sufficient co-enzyme present to give the maximum activity.

When the reduction time is greater than 35 minutes there is a rather rapid falling off in activity even in the presence of heated bacteria. This effect might well be due to the gradual poisoning of the enzyme by methylene blue, which was noticed above.

It is evident, therefore, that there is present in *Bact. coli* a thermostable substance capable of acting as co-enzyme to glucose dehydrogenase. The properties of this co-enzyme are now being investigated.

DISCUSSION.

The methylene blue technique as used for the study of bacterial dehydrogenases has been shown to be limited in its applicability. Unless the reduction time is taken between certain limits, which vary for the various dehydrogenases, the activity measured is not a true representation of the strength of the enzyme. For example, with succinic and formic dehydrogenases, when the reduction time is less than 20 minutes, there is an error due to the time taken for the temperature of the reactants to reach that of the bath. The activity is thus too low. This error necessitates a correction to many of the results quoted in the literature.

The upper limit of reduction time for proportionality to hold varies considerably for the different enzymes. Formic dehydrogenase shows a constant "activity $\times$ dilution" up to very long reduction times. Only when the reduction exceeds 2 hours is there a falling off in the activity, and this is very slight. These results are in accordance with the stability of this enzyme to methylene blue and temperature, and the absence of effect of the phosphate buffer.

The results with succinic dehydrogenase, which shows a decreasing activity after 50 minutes, have been explained by the poisoning effect of methylene blue. The accelerating effect due to the action of the organisms on the succinate itself is not sufficient to compensate for this poisoning by the dye.

Glucose dehydrogenase, although poisoned to a greater extent than succinic dehydrogenase by methylene blue, shows a very striking lack of proportionality between activity and dilution. It was found that there is present a thermostable co-enzyme in the bacteria which is probably washed out on dilution. On the addition of a heated suspension of bacteria, the effect of dilution is what one would expect from the poisoning of the enzyme by methylene blue. Here, as with succinic dehydrogenase, it would seem that the poisoning by the dye outweighs the acceleration due to the decomposition of glucose.

SUMMARY.

1. The effect of dilution on activity has been studied for three enzymes of *Bact. coli*, the formic, succinic and glucose dehydrogenases. The product of the activity and dilution, which should be constant, is found to be so within certain limits for the first two of these but not at all for the glucose enzyme.

2. The various factors involved in the methylene blue technique have each been considered in an attempt to explain these discrepancies.

3. None of the enzymes is affected by being kept at 40° for one hour.

4. Formic dehydrogenase is unaffected by being kept for 5 hours with methylene blue or one hour with phosphate buffer.

5. Succinic dehydrogenase is slowly poisoned to a maximum of 40 % by methylene blue. Its action is accelerated when it is kept in presence of phosphate buffer or with succinate.

6. Glucose dehydrogenase is poisoned to 80 % by methylene blue. Its action is accelerated when it is kept with phosphate buffer or with glucose.

7. It is shown by treatment with toluene, silver *etc.*, that the accelerating effect on glucose dehydrogenase produced when the bacteria are kept in contact with glucose is due to the production of substances from the glucose which are activated by the bacteria as hydrogen donators.

8. A thermostable co-enzyme of glucose dehydrogenase has been demonstrated in *Bact. coli*.

9. The effects of dilution on the three enzymes can be explained by the properties of the enzymes studied in this paper.

10. The limitations of the methylene blue technique are discussed.

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