CXXV. FORMIC DEHYDROGENASE OF *BACTERIUM COLI*: ITS INACTIVATION BY OXYGEN AND ITS PROTECTION IN THE BACTERIAL CELL

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QUASTEL & WHETHAM [1925] showed that washed suspensions of *Bact. coli* will reduce methylene blue in the presence of formate and that the rate of reduction under standard conditions is greater with formate as substrate than for any other substance, with the exception of the sugars. Stickland [1929] showed that if a suspension of the organism is incubated with *liquor pancreaticus* then the activity of the formic dehydrogenase with methylene blue increases until it reaches 4 or 5 times its original value after several days. The activity of other dehydrogenases also increases at first but then rapidly decreases until the preparation is eventually active only with formate. After filtration of this preparation through a glass filter, 50–60 % of the activity is found to reside in the filtrate which contains large quantities of cell debris. When these are removed by high speed centrifuging, the supernatant fluid is almost inactive. Although the original bacterial suspension is able to oxidize formate to completion, the dehydrogenase preparation is unable to react with O_2 in the presence of formate. A similar phenomenon was encountered by Stephenson [1928] with a preparation of lactic dehydrogenase from *Bact. coli* but, in that case, the preparation was able to utilize O_2 as H-acceptor in the presence of a trace of methylene blue as carrier. Stickland was unable to obtain an O_2 uptake with his preparation by the addition of 0·5 ml. methylene blue (1/5000) but obtained, with or without the dye, a small uptake amounting to 20–30 μl. of O_2. He was unable to find any explanation of this effect and could only obtain complete oxidation of formate by, first, the anaerobic reduction of a large quantity of methylene blue by the formate in the presence of the preparation, followed by the aerobic re-oxidation of the reduced methylene blue.

In the course of studies of the enzyme systems of *Bact. coli* by investigation of the material obtained by crushing cell suspensions in the wet-crushing mill of Booth & Green [1938] it was decided to reinvestigate this problem, with the results set out in the following paper.

Technique. The organisms in all cases were grown on the surface of broth-agar in Roux bottles for 24 hr. They were then washed off with distilled water, spun out, washed twice in water and finally suspended in distilled water. The strength of such suspensions was determined by means of a photoelectric turbidimeter [Clifton *et al.* 1935]. The Q_{O_2} (μl. O_2 taken up/hr./mg. dry weight of organism) was determined in Warburg manometers; 1 ml. each of the bacterial suspension, $M/40$ Na formate and $M/10$ phosphate buffer at *p*H 6·0 being placed in the main compartment of the manometer cup and 0·2 ml. 10 % NaOH in the centre cup. All values of Q_{O_2} are corrected for the blank respiration of the organism. The activity with methylene blue was determined in Thunberg tubes evacuated and incubated at 37°. The following quantities were used: 1 ml. bacterial suspension, 1 ml. $M/10$ Na formate (in the hollow stopper) and 1 ml.

phosphate buffer at *p*H 6·0, in the presence of 0·2 ml. of 0·5 % methylene blue. The substrate was tipped into the reaction mixture after equilibration and the time (*T* sec.) taken to reduce the dye to completion was noted. The activity of the dehydrogenase towards methylene blue is given as Q_{MB}, defined as: $Q_{MB} = \mu$l. O_2 equivalent to the methylene blue reduced/hr./mg. dry weight of preparation. Under the experimental conditions described, this is equal to $108{,}000/TW$, where W = dry weight of preparation.

For the preparation of cell-free material from the organisms, 30–40 Roux bottles were inoculated with the organism and incubated for 24 hr. The total quantity of cells so obtained was suspended, after washing, in 50 ml. of water and then ground for 2½ hr. in the Booth-Green mill. The cream obtained in this manner was investigated for formic dehydrogenase activity as below.

Distribution of the formic dehydrogenase in the crushed material

The activity of the ground material as formic dehydrogenase was investigated by the methylene blue technique and the results compared with the activity of the original washed suspension of organisms. Next, the ground material (1) was centrifuged for 30 min. at 3000 r.p.m.; this treatment provides two fractions: a brownish-yellow opaque supernatant fluid (2) and a thick sediment (5). The material (2) was then further centrifuged at 11,000 r.p.m. for 20 min. and gave a clear yellow fluid (3). Finally, this clear fluid was filtered through a Seitz filter, giving a water-clear fluid (4). The dry weight of each fraction was determined by drying suitable amounts in tared vessels to a constant weight in a steam oven and the methylene blue activity (Q_{MB}) then determined as above. The results are set out in Table I.

Table I. *Distribution of formic dehydrogenase in crushed material*

Material	Q_{MB} Exp. 1	Q_{MB} Exp. 2
Washed suspension of organism	183	118
Ground material (1)	104	72
Opaque supernatant fluid (2)	38	25
Clear yellow fluid (3)	13	9
Filtered fluid (4)	0	0
Washed sediment (5)	218	136
Dried sediment	45	11

It is obvious from these results that the formic dehydrogenase activity resides in the solid particles, as fractional removal of these leads to a steady loss of activity, the final filtered fluid being inactive. Accordingly further work was carried out on the washed sediment (1) only. Attempts to remove the enzyme from the surface of the solid material by (i) extraction with phosphate buffers of *p*H 5–10 either before or after digestion with trypsin, (ii) digestion with trypsin, (iii) digestion with papain etc. were completely unsuccessful.

Oxidation of formate by the sediment. Reference to Table II shows that the sediment gives a Q_{O_2} of approximately the same value as that obtained with the whole organism but its Q_{MB} has decreased below that for the organism. The sediment incubated under the usual conditions with 1 ml. of *M*/40 formate gives a total oxygen uptake of 288 μl. so that the oxidation is taken to completion in agreement with the equation:

$$H.COOH + O = H_2O + CO_2 \text{ (theor. as above} = 280\,\mu\text{l.)}$$

This reaction has been established for the whole organism by Cook & Stephenson [1928].

Fig. 1 shows the effect of pH on the Q_{MB} and the Q_{O_2}. The former is more sensitive to changes in pH than the oxidation system. A working pH of 6·0 has been adopted for all experiments.

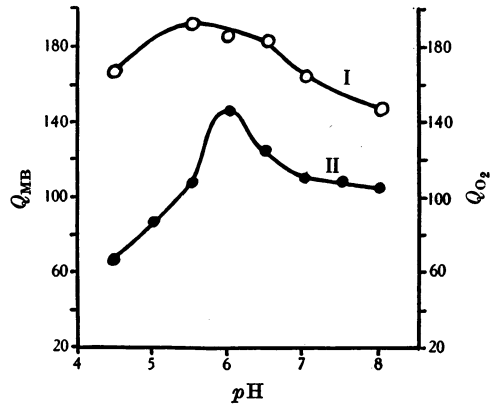

Fig. 1. Effect of pH on Q_{O_2} (I) and Q_{MB} (II) with formic dehydrogenase of the sediment.

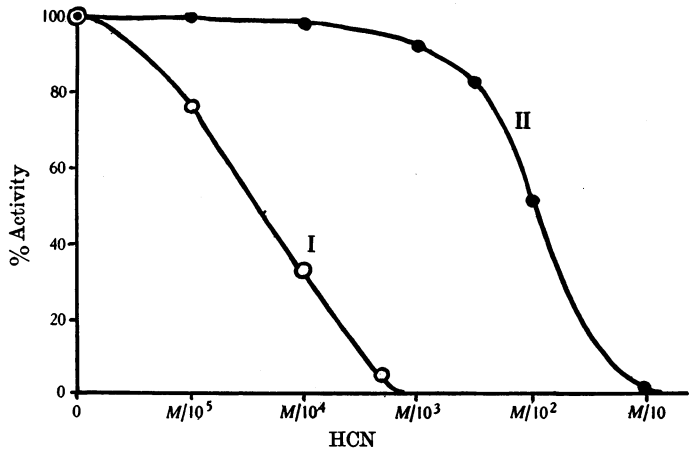

Fig. 2. Effect of HCN on Q_{O_2} (I) and Q_{MB} (II) of formic dehydrogenase in sediment.

Fig. 2 shows the effect of cyanide on the Q_{MB} and Q_{O_2}. The O_2 uptake is completely inhibited by a concentration of $M/1000$ cyanide but this concentration inhibits the methylene blue activity by 8 %, complete inhibition of the dehydrogenase only being obtained with $M/20$ cyanide. During the Q_{O_2} determinations mixtures of alkali and cyanide were used in the centre cup according to the technique of Krebs [1935]. From these results it appears that formic dehydrogenase differs from most dehydrogenases in being inhibited by cyanide. The preparation inactivated in the presence of $M/10$ cyanide is not restored by washing three times in 1000 times its weight of water so that the inhibition would appear to be irreversible. Adler & Svreenivasaya [1937] have also shown that formic dehydrogenase extracted from peas is inhibited by cyanide.

Effect of digestion of the sediment with trypsin. The sediment was next incubated in the presence of phosphate buffer at *p*H 7·5 with trypsin. 1 % fluoride was used as antiseptic as this has no effect upon the dehydrogenase activity. The trypsin preparation used was the "Pangestin" preparation of "Difco", in a concentration of 10 % of the weight of material to be digested. At intervals samples were withdrawn, washed thoroughly until free from trypsin, the dry weight determined and the Q_{MB} and Q_{O_2} investigated. Also the total O_2 uptake obtained in the presence of 1 ml. $M/40$ formate (theory = 280 μl.) with an amount of material equivalent to 2 mg. of the original sediment was determined. The results are given in Table II.

Table II. *Effect of digestion of sediment with trypsin*

Material	Digestion time	Q_{O_2} formate	Q_{MB}	Total O_2 uptake (μl.)
Washed suspension of organism	—	171	158	?
Washed sediment	—	166	107	290
	18 hr.	152	226	156
	40 hr.	132	366	102
	60 hr.	118	334	72
	6 days	114	603	34
	7 days	35	475	15
	8 days	—	442	0

From these figures it is seen that:

(1) The initial Q_{O_2} falls steadily but slowly for 6 days and then falls rapidly to zero by the 8th day.

(2) The Q_{MB} rises steadily until the 6th day and then falls but has 300–400 % of the original activity when $Q_{O_2} = 0$.

(3) The total O_2 consumption decreases rapidly as digestion proceeds.

During this digestion the dry weight of the sediment decreases to 25 % of its original value and the rise in Q_{MB} is largely accounted for by the decrease in dry weight due to removal of inactive material. However, in some cases, an increase in actual dehydrogenase activity amounting to some 200 % has been observed—as found by Stickland [1929]. Further digestion after the 8th day in the presence of fresh trypsin leads to a slow but steady loss in dehydrogenase activity.

Sediment allowed to autolyse in the presence of fluoride at 37° showed the changes set out in Table III. The experiments were all carried out with an amount of material equivalent to 2 mg. of the original sediment and the results show an increase in Q_{MB} while the Q_{O_2} remains unchanged but the total oxygen uptake is limited to 100–150 μl. after 6 days' autolysis.

Table III. *Effect of autolysis of sediment*

Material	Autolysis time	Q_{O_2} formate	Q_{MB}	Total O_2 uptake (μl.)
Washed suspension of organism	—	171	158	?
Washed sediment	—	166	107	290
	20 hr.	210	281	174
	40 hr.	184	281	154
	5 days	182	356	141
	6 days	153	324	137

It will be convenient to refer, in the following experiments, to the preparations as follows:

Material A. The untreated, washed sediment.

Material B. The sediment after 7 days' autolysis, able to oxidise 1 ml. $M/40$ formate to 50 % completion.

Material C. The sediment after 7 days' digestion, with a high Q_{MB} but unable to react with O_2.

In all cases a quantity of material, in suspension, was used equivalent to 2 mg. dry weight of material A. The materials were kept in water suspension in the presence of 1 % fluoride in the ice-chest; for use they were spun out, washed and resuspended in water. Desiccation of the materials leads to a marked loss of activity, see Table I.

It appears from the results obtained that the material C contains the formic dehydrogenase but that this is unable to react with O_2 in the absence of some factor which has been removed by the digestion with trypsin. This would also explain the differences between the methylene blue and O_2 activities in Figs. 1 and 2.

Spectroscopic examination of the materials. A sample of material A containing 50 mg./ml. was examined under the microspectroscope. No absorption bands were visible and slight warming produced no alteration. When hydrosulphite was added, the bands of cytochrome *b* were easily visible. On shaking the material with air the bands disappeared again. Next, formate was added to a fresh sample and, after momentary warming, the bands appeared strongly. The addition of $M/10{,}000$ cyanide had no effect on either the reduction of the cytochrome by formate or the reoxidation by shaking with air. $M/50$ cyanide inhibited the reduction of the cytochrome in the presence of the formate but not by hydrosulphite. This shows, from Fig. 2, that the formic dehydrogenase of the sediment can react with O_2 through the cytochrome system.

Examination of a similar sample of material B gave similar results except that the appearance of the cytochrome bands was slower in this case when the material was reduced with formate.

Material C, on the other hand, appeared to contain little or no cytochrome as no absorption bands appeared either on warming with formate or reduction with hydrosulphite. When a preparation of cytochrome *c*, extracted from heart muscle, was added to material C in the presence of formate and warmed, the spectrum of reduced cytochrome *c* appeared and the bands remained until a preparation of cytochrome oxidase from heart muscle was added, when the bands disappeared on shaking with air. Thus it would appear that the cytochrome *c*—cytochrome oxidase system can act as a carrier system in conjunction with the formic dehydrogenase of material C.

However, when material C was shaken in a Warburg manometer with phosphate buffer, formate and 0·2 ml. of a 0·06 % cytochrome *c* preparation and cytochrome oxidase, no significant O_2 uptake could be obtained. Further, material B, which contains cytochrome, is unable to oxidize 1 ml. of $M/40$ formate to completion but gives an O_2 rate which falls off steadily to zero after about an hour. The effect appears to be due to inactivation of the enzyme system and this was the next effect studied.

Inactivation of material B. Fig. 3 shows the oxygen consumption under standard conditions with varying initial concentrations of formate and 1 mg. (dry weight) of material B. Table IV shows the percentage theoretical uptake obtained in each case together with the initial Q_{O_2} determined for the first 10 min. of the reaction. The higher the initial formate concentration, the higher the initial Q_{O_2} but the lower the percentage oxidation of substrate.

Stickland [1929] stated that the possible by-products of the dehydrogenation, oxalic acid and formaldehyde, do not affect the reaction until their concentration

reaches a higher level than could be formed from the formate present. This was confirmed, $M/100$ oxalate or $M/100$ H.CHO having no effect.

Particular attention was paid to the possibility of the formation of H_2O_2 and this was ruled out from the following considerations:[1]

(1) All the preparations contain powerful catalase activity.

(2) The addition of alcohol in an attempt to obtain a coupled oxidation in the presence of catalase [Keilin & Hartree, 1936] has no effect on the O_2 consumption. Further, tests for acetaldehyde after such experiments proved negative. The test used was the fuchsin sulphurous acid spot test which, according to Feigl [1937], will detect 4μg. of acetaldehyde.

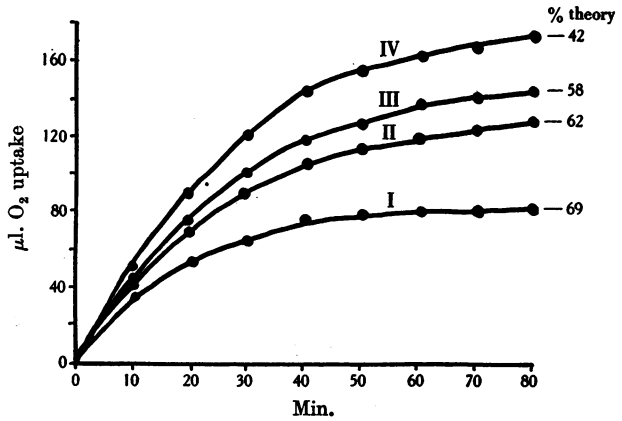

Fig. 3. Effect of initial formate concentration on inactivation, see Table IV. Initial concentration: I, $M/240$ formate. II, $M/160$ formate. III, $M/120$ formate. IV, $M/80$ formate.

Table IV. *Effect of initial formate concentration on inactivation*

ml. of $M/40$ formate added	Theoretical O_2 uptake μl.	Actual O_2 uptake μl.	% uptake	Initial Q_{O_2}
0·5	140	96	69	142
0·75	210	131	62	148
1·0	280	165	58	208
0·75 ($M/20$)	420	175	42	279

(3) No colour can be obtained, after a reaction, with guaiacum and peroxidase.

(4) $M/3000$ H_2O_2, easily detectable by the above test, has no effect on the Q_{O_2} or on the total O_2 consumption.

(5) The presence of peroxidase and *p*-phenylenediamine during the reaction has no effect on the Q_{O_2} or on the total O_2 consumption.

(6) In (5) slight browning of the reaction mixture occurred during the experiment but there was no difference in the depth or rate of appearance of the colour in the experiment and in the control without formate.

In case the effect should be due to a loss or deficiency of some factor in the oxidation carrier system, the addition of the following substances was tested: cozymase, lactoflavin, cytochrome oxidase, cytochrome *c* (0·2 ml. of 0·06 % solution, see p. 1023), boiled organism, malic and fumaric acids. The inactivation was not altered or checked by any of these additions or by combinations of these substances.

[1] See also Bhagvat & Hill (in preparation).

Conditions for inactivation. Fig. 4 shows the O_2 uptakes in two manometers containing material B in the usual amount. In manometer 1 (curve 1) 0·5 ml. of $M/40$ formate was tipped into the reaction mixture at zero time and the reaction allowed to proceed until the preparation was inactivated after 70 min. Then a further 0·5 ml. of $M/40$ formate was tipped in: the preparation was then unable to oxidize the freshly added substrate. In manometer 2 (curve 2) no formate was added at first but the enzyme preparation was shaken with buffer in air under similar conditions to those in manometer 1. Then 0·5 ml. of $M/40$ formate was added at the same time as the second addition to manometer 1. In this case the enzyme was able to oxidize the formate readily.

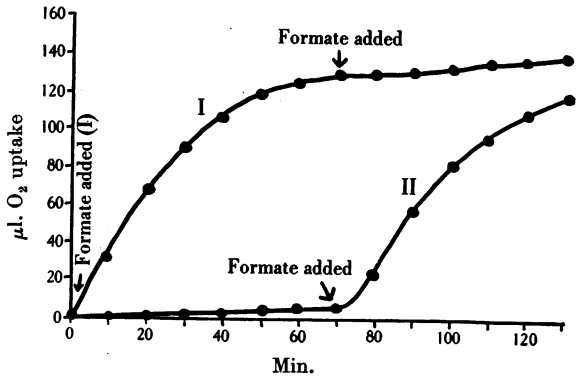

Fig. 4. Effect of presence of substrate on inactivation, see text.

This experiment shows that the enzyme is not inactivated by shaking in air under the experimental conditions but that the presence of the substrate is essential for the inactivation to occur. It also disposes of the supposition that the falling off in the rate of oxidation might be due to the establishment of an equilibrium since, in manometer 1, the addition of further reactant has no effect upon the course of the reaction.

In this experiment, the Q_{MB} of the preparation at the beginning of the experiment was 475. After shaking in air alone at 37° for 70 min., the value of the Q_{MB} was unchanged but the enzyme which had been incubated aerobically with the formate for the same time was completely inactivated. Thus the inactivation applies not only to the O_2 uptake but also to the methylene blue activity so that it is the dehydrogenase itself which is inactivated.

In order to determine whether the enzyme would be inactivated anaerobically in the presence of its substrate, 1 ml. of preparation containing 1 mg. dry weight of material B was incubated in a Thunberg tube with 1 ml. of $M/20$ formate and 1 ml. of phosphate buffer at *p*H 6; 2 ml. of 0·5 % methylene blue were tipped in slowly from the hollow stopper as the reduction progressed in the evacuated tube. The whole of the dye was reduced, being equivalent to an O_2 consumption of 300μl., and the Q_{MB} of the material was unchanged. A similar experiment carried out aerobically in the absence of methylene blue gave an oxygen consumption of 113μl., after which the enzyme was completely inactivated, having a Q_{MB} of less than 10.

Thus the dehydrogenase is inactivated only if it is incubated aerobically in the presence of its substrate.

Effect of incubation with glutathione (GSSG). Hopkins & Morgan [1938] have shown that the activity of succinic dehydrogenase of animal tissues is completely abolished by incubation with GSSG. The inactive material can then be reactivated by incubation with GSH and the authors suggest that the activity of the enzyme may depend upon the presence of certain SH groups. In order to test whether any such effect could be obtained with the formic dehydrogenase preparation, the following experiment was carried out: a series of Thunberg tubes were made up as set out in Table V and then incubated anaerobically for the times indicated. After this period the material was centrifuged out, washed, resuspended in water and the reduction time determined as usual in each case. It is seen from Table V that GSSG has no effect on the activity in the absence of formate but produces a slow decrease of activity when incubated with the dehydrogenase in the presence of its substrate.

Table V. *Effect of GSSG on formic dehydrogenase*

	Preliminary incubation. ml. of additions					
Tubes	1	2	3	3*a*	4	4*a*
Material B (2 mg./ml.)	2	2	2	2	2	2
M/20 phosphate	2	2	2	2	2	2
M/10 formate	—	1	—	—	1	1
M/50 GSSG	—	—	1	—	1	—
M/10 GSSG	—	—	—	1	—	1
Water	2	1	1	1	—	—

Activity after incubation

	Reduction times after	
Material from tube no.	2 hr. incubation min. sec.	1 hr. incubation min. sec.
1	7 0	6 30
2	7 10	6 30
3	7 5	6 30
4	8 5	8 30
3*a*	—	6 30
4*a*	—	10 30

Reactivation of inactivated enzyme. 10 ml. of a suspension of material B were incubated in the presence of 10 ml. *M*/10 formate and 10 ml. *M*/20 phosphate buffer at *p*H 6 for 2 hr., being bubbled vigorously with air throughout the incubation period. At the end of that time, a sample was taken and its Q_{MB} determined. The remainder of the material was centrifuged out, washed once and made up to 9 ml. with water. 1 ml. was placed in each of eight Thunberg tubes and the various additions indicated in Table VI then made. The tubes were incubated, 1 and 3 aerobically and the rest anaerobically, for 1 hr. In the hollow stoppers 1 ml. *M*/10 formate and 0·2 ml. 0·5 % methylene blue (the latter omitted in tubes 3 and 4) were placed and after the preliminary incubation, the contents of the stoppers were tipped in, all the tubes evacuated and the Q_{MB} determined in each case. From Table VI it is seen that anaerobic conditions in the presence or absence of formate succeed in reactivating the enzyme. GSH has no significant effect.

Fig. 5 shows the results of an experiment in which the rate of reactivation of a partially inactivated preparation was studied under anaerobic conditions and in the presence of formate anaerobically, by means of an experiment similar to the last in which the contents of the stoppers were added at 30 min. intervals. The presence of the formate accelerates the reactivation process. Since the inactivation process occurs only in the presence of formate, it is possible that the

Table VI. *Reactivation of inactivated dehydrogenase*

		Q_{MB} after treatment
	Original material ...	442
	After aerobic incubation with formate	43
	Conditions during preliminary incubation	
Tube	Additions	
1	1 ml. water (aerobic)	43
2	1 ml. water (anaerobic)	366
3	1 ml. water 0·2 ml. 0·5 % MB (aerobic)	43
4	1 ml. water 0·2 ml. 0·5 % MB (anaerobic)	345
5	1 ml. *M*/100 GSH (anaerobic)	172
6	1 ml. *M*/100 GSSG (anaerobic)	108
7	1 ml. boiled organism (anaerobic)	21
8	1 ml. *M*/10 formate (anaerobic)	377

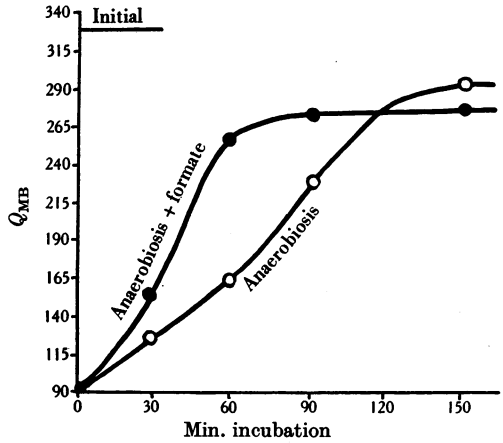

Fig. 5. Reaction of partially-inactivated enzyme by incubating, (*a*) anaerobically; (*b*) anaerobically with formate.

effect of anaerobic conditions alone in this last result may be due to the presence of unremoved traces of formate. It has not been possible to establish this as prolonged washing of the inactivated material renders it permanently inactive.

It would seem likely that the reactivation process is one of reduction. Moreover, the conditions necessary for the inactivation of the enzyme in the first place make it appear that the inactivation is associated with an oxidation of the dehydrogenase during its action with its substrate. Reference to Table II shows that the enzyme becomes more susceptible to the inactivation process as digestion of the sediment progresses. Further, it can be shown that the cytochrome content of the sediment decreases during digestion until material C is devoid of cytochrome. The question then arises as to whether the cytochrome normally "protects" the enzyme from inactivation by oxidation and this point will now be considered.

Effect of O_2 tension on the inactivation of material B

A series of manometers were set up with the usual contents for the investigation of the Q_{O_2} of material B. These were then gassed with O_2-N_2 mixtures containing the following percentages of O_2: 100, 50, 20, 10, 5, $2\frac{1}{2}$. Fig. 6 shows the O_2

uptakes followed over 10 min. intervals. In pure oxygen, the enzyme is quickly inactivated, the oxygen uptake being reduced to zero in 30 min. As the percentage of O_2 in the gas phase decreases so does the rate of inactivation until, in $2\frac{1}{2}$ % O_2, the O_2 consumption is linear for at least 2 hr.

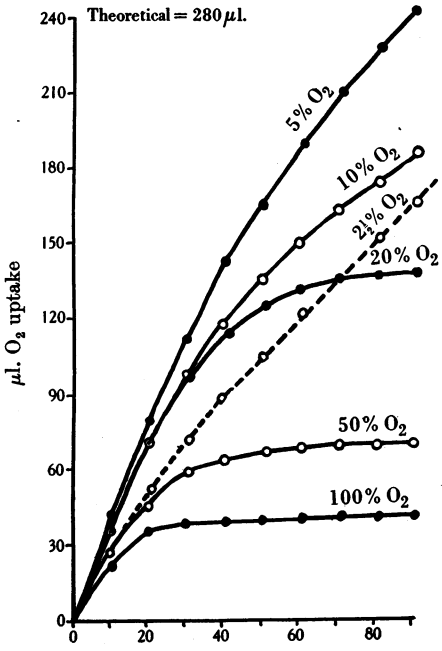

Fig. 6.

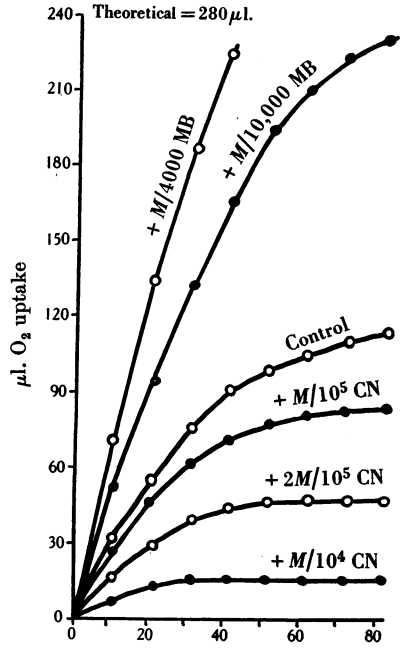

Fig. 7.

Fig. 6. Effect of oxygen tension on inactivation of formic dehydrogenase in material B.

Fig. 7. Effect of (*a*) MB; (*b*) CN on inactivation of formic dehydrogenase of material B in air.

Next the effect of altering the amount of potential oxygen-carriers in the system was tried. The rate of reaction of the dehydrogenase with O_2 through carrier systems can be increased by the addition of methylene blue, or decreased, if the natural carrier present is cytochrome as indicated by the spectroscopic observations, by the action of cyanide. Fig. 7 shows the effect of adding methylene blue to final concentrations of $M/4200$ and $M/10{,}500$ and of cyanide to $M/10^5$, $2M/10^5$ and $M/10^4$, the gas phase in all cases being air. Increasing the velocity of the reaction of the dehydrogenase with O_2 through carrier systems results in an inhibition of the inactivation process; while the action of cyanide, which inhibits the reaction through the cytochrome system, speeds up the inactivation of the dehydrogenase.

These effects can be reconstructed using methylene blue as the sole carrier with material C which contains no natural carrier. The material C when shaken with formate aerobically gives an O_2 uptake of 5–12 µl. and is then inactivated towards either O_2 or methylene blue. Fig. 8 shows the effect of adding various concentrations of MB to material C and then shaking with formate in air. Low concentrations of the dye—less than $M/10^5$—have very little effect but higher concentrations enable O_2 to be utilized to an extent depending upon the

dye concentration: the higher this concentration, the slower the inactivation of the dehydrogenase.

Fig. 9 shows the course of the O_2 uptake by material C in the presence of formate and a constant amount of MB, $M/4200$, but with various O_2 tensions. High percentages of O_2 in the gas phase lead to a rapid inactivation of the dehydrogenase while if the O_2 tension is sufficiently reduced, then a linear uptake is obtained over the experimental period. The general picture is thus similar to that of Fig. 6 in which the carrier system is cytochrome.

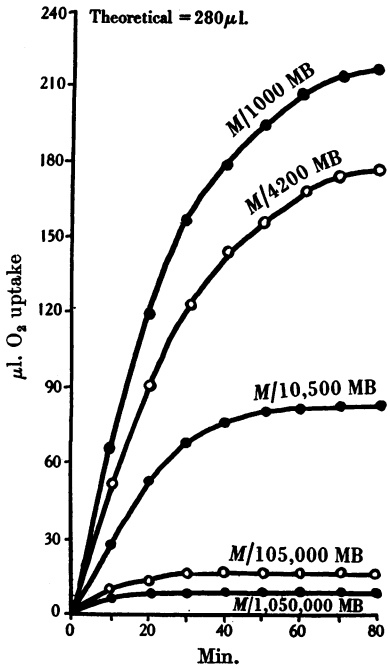

Fig. 8.

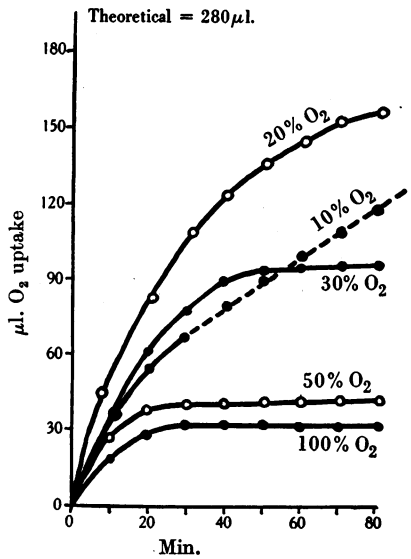

Fig. 9.

Fig. 8. Oxygen uptake with material C in presence of formate and varying concentrations of MB in air.

Fig. 9. Course of O_2 uptake with material C in presence of formate, $M/4200$ methylene blue and varying oxygen tensions.

In every case, the rate of inactivation of the dehydrogenase is accelerated by decreasing the concentration of the oxygen-carrying systems present, but is inhibited by increasing their concentration. In the presence of a constant amount of carrier, then the rate of inactivation is dependent upon the O_2 tension; the higher the tension, the more rapid the inactivation process.

It would appear that the enzyme can react with O_2 along two paths—directly, leading to inactivation of the dehydrogenase activity, and indirectly through a carrier system; which process predominates depends upon (*a*) the O_2 tension and (*b*) the amount of carrier present. Increasing the O_2 tension, while the amount of carrier present is unchanged, would speed up the direct reaction and thus the inactivation process; while increasing the amount of carrier favours the indirect reaction and so diverts the O_2 from the inactivation process. Under physiological conditions it is probable that the reaction through the natural

carrier system is so rapid that the O_2 is diverted from the direct reaction and inactivation proceeds only slowly. This is dealt with later.

Reconstruction of the cytochrome system. Previously it has been impossible to obtain an O_2 uptake by material C in the presence of formate by the addition of cytochrome *c* and cytochrome oxidase. It would appear that this result may be due to (*a*) insufficiently high concentration of cytochrome and (*b*) too high O_2 tension. Also it must be remembered that cytochrome *c* is not the natural cytochrome involved but no method of preparing the cytochrome *b* of bacteria is yet available. Hence manometers were set up with the following additions:

(1) and (3) 0·5 ml. 1 % cytochrome *c*; 0·3 ml. cytochrome oxidase.

(2) 0·25 ml. 1 % cytochrome *c*; 0·2 ml. cytochrome oxidase.

Manometers 1 and 2 were filled with O_2-N_2 mixtures containing 4 % O_2 and manometer 3 was filled with air. On incubation with formate, and material C, manometer 3 gave an uptake of 10 μl. in 30 min. after which the enzyme was inactive. Manometer 1 showed a linear O_2 uptake lasting for 2½ hr. before the experiment was stopped: the steady $Q_{O_2} = 29$. Manometer 2 also showed a small O_2 uptake ($Q_{O_2} = 13$) which decreased slowly after 90 min. None of the controls from which any of the components (enzyme-formate-cytochrome-cytochrome oxidase-oxygen) was missing showed any significant activity. (I am indebted to Dr Hartree of the Molteno Institute, Cambridge, for the concentrated solution of cytochrome *c* and the cytochrome oxidase preparations.)

This reconstruction proves that the formic dehydrogenase of *Bact. coli* reacts with O_2 through the cytochrome system.

Effect of oxygen on the formic dehydrogenase of Bact. coli *grown in air.* A culture of *Bact. coli* was grown on the surface of broth agar in air for 20 hr., washed twice and resuspended in distilled water; dry weight of suspension = 1·2 mg./ml. Manometers were then set up containing 1 ml. bacterial suspension, 1 ml. phosphate buffer *p*H 6 and 1 ml. *M*/40 formate (water in controls); the manometers were filled with O_2-N_2 mixtures containing air, 30, 50, 100 % O_2 respectively. The course of the O_2 uptake at 37° under these conditions is set out in Fig. 10. An oxygen tension greater than that in air has an inactivation effect depending upon its tension. In order to investigate this further, two terms are defined:

Critical oxygen tension: the maximum O_2 tension at which the rate of O_2 consumption is not more than halved in 1 hr.

Protection index: the number of minutes in which the O_2 consumption is halved when the system is acting in 100 % O_2.

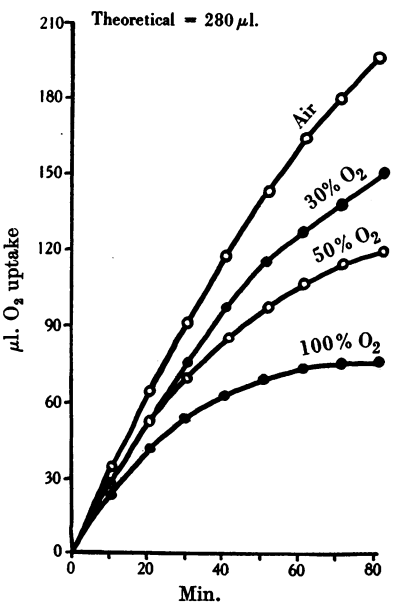

Fig. 10. Oxidation of formate by *Bact. coli* grown in air—effect of O_2 tension on oxygen uptake.

For the organism grown in air, the critical O_2 tension is that holding in air, 21 %, and the protection index, from Fig. 10, is 25. Both the critical O_2 tension and the protection index are measures of the degree of protection afforded to the enzyme by the carrier systems present but, since they are determined by

separate experiments, are mutually corroborative. The relevant facts for materials A, B and C are set out in Table VII.

Table VII

Material	Q_{O_2}	Q_{MB}	Cytochrome content	Critical O_2 tension	Protection index
Untreated sediment A	164	110	× × ×	15–20 %	20
Autolysed material B	153	324	×	15 %	15
Digested material C	0	474	—	0	0

Effect of the oxygen tension present during growth of the organism

In order to grow cultures on the surface of agar under any desired O_2 tension, the following technique was devised: a Roux bottle containing broth-agar is sterilized and, while the agar is still liquid, a small sterile glass boat is slipped in through the neck and embedded in the agar. When cool, the agar is inoculated as usual and 0·5 ml. 10 % NaOH put in the boat. The cotton-wool plug is replaced by a sterile rubber bung fitted with an inlet tube plugged with sterile cotton-wool. The bottle is then connected to a three-way tap connected, on one side to a pump and on the other to an aspirator containing the desired gas mixture. The Roux bottle is then filled with the gas mixture by partial evacuation followed by connexion with the aspirator. If the bottle is one-third evacuated each time and the complete operation repeated some 15 times, almost complete exchange will be effected. When full the bottle is clamped off, disconnected and then reconnected to an aspirator containing O_2. A water-valve is put in between to prevent mixture of the gases. The whole apparatus is now incubated and as O_2 is used up by the growing organisms, the evolved CO_2 will be absorbed by the NaOH in the boat and O_2 will be sucked in from the aspirator to keep the O_2 tension constant.

In this manner cultures have been grown in the presence of O_2-N_2 mixtures containing 100, 80, 50, 20 and 0 % O_2. In every case, the cultures have been

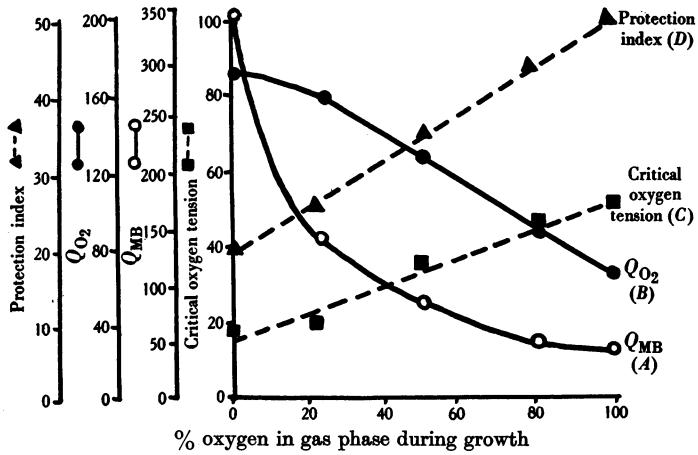

Fig. 11. Effect of oxygen tension present during growth on the properties of *Bact. coli* in regard to formate oxidation. *A*, Q_{MB}; *B*, Q_{O_2}; *C*, critical oxygen tension; *D*, protection index.

prepared in washed suspension as usual and the following quantities determined: Q_{MB}; Q_{O_2} in air during linear O_2 consumption over the first 20 min., critical O_2 tension and protection index. The results are plotted in Fig. 11 against the O_2 percentage during growth.

It is seen that increasing the O_2 tension present during growth leads to (1) a decrease in dehydrogenase activity as shown by the drop in Q_{MB} correlated with (2) a decrease in Q_{O_2} and (3) an increase in both critical O_2 tension and protection index; Wooldridge *et al.* [1936] have shown that the formic dehydrogenase of *Bact. coli* undergoes negligible variation with the age of the culture so that the results are not complicated by any such effects.

A rise in the critical O_2 tension and the protection index would be brought about, from what has been shown, by a variation in the ratio of carrier systems/enzyme present. Since the cytochrome bands for the organism are weak, it has proved impossible to state whether the cytochrome content of the organism varies significantly according to the manner of growth. However, a culture that has been grown through 20 consecutive anaerobic subcultivations appears to contain essentially the same amount of cytochrome as one grown aerobically for the same period. (I am indebted to Dr T. Mann of the Molteno Institute, Cambridge, for the examination of various cultures with respect to their cytochrome contents.) If, as seems probable, the cytochrome content of the organism is approximately constant then it would follow that any rise in the critical O_2 tolerance would be brought about by inhibition of dehydrogenase activity. From the appearance of curves *A* and *C* (Fig. 11) this would appear to be the case.

When the organism is grown anaerobically, a very high Q_{MB} is found. This is not due to the presence of formic hydrogenlyase in the anaerobic culture reducing MB through the hydrogenase system as formic hydrogenlyase is not active at the experimental *p*H 6 used [*v.* Stephenson & Stickland, 1932]. Hence the high Q_{MB} must be due to a greatly increased production of the dehydrogenase under anaerobic conditions.

Absence of coenzymes I and II in material C. Adler & Svreenivasaya [1937] showed that the formic dehydrogenase of certain plants requires the presence of coenzyme I for its action. Table VIII shows the effect of adding coenzymes I and

Table VIII. *Effect of addition of coenzymes on activity of formic dehydrogenase in material C*

Tubes contain: 1 ml. *M*/10 Na formate.
1 ml. *M*/20 phosphate buffer *p*H 6·0.
1 ml. material C = 0·45 mg. dry weight.
0·2 ml. 0·5 % methylene blue.

Additions	Reduction time min.	sec.
1 ml. water	9	30
1 ml. cozymase (=0·15 mg. coenzyme I)	9	30
1 ml. coenzyme II prep. (=0·2 mg. coenzyme II)	9	20
1 ml. boiled organism (=20 mg. dry wt.)	12	0

II and boiled organism on the Q_{MB} of material C in the presence of formate. There is no significant acceleration. Gale & Stephenson [1939] have shown that the malic dehydrogenase of *Bact. coli* requires coenzyme I but that the organism grown on agar possesses only about 27 % of the optimal amount of coenzyme required to activate the dehydrogenase present—hence, in this case, addition of cozymase to the washed suspension produces a considerable acceleration of the rate of reduction of methylene blue. No such effect is obtained with formic dehydrogenase. Further, Yudkin [1933] has shown that the activity of formic dehydrogenase of *Bact. coli* does not vary with the dilution of the organism whereas the activity of other dehydrogenases, known to require coenzymes,

decreases rapidly with dilution. Thus it would appear that the activity of formic dehydrogenase of *Bact. coli* does not depend upon the presence of a coenzyme. Table IX shows the effect of adding 1 ml. of boiled material C containing 20 mg. dry weight to (*a*) malic dehydrogenase from heart muscle and from *coli*-juice [Gale & Stephenson, 1939] and (*b*) glucosemonophosphate dehydrogenase from muscle. Also the effects of adding known amounts of coenzymes I and II and boiled *Bact. coli* (20 mg. dry weight) are shown. It is obvious that material C contains no coenzyme I or II. Hence the formic dehydrogenase of *Bact. coli* does not require either coenzyme for its action.

Table IX. *Absence of coenzymes I and II from material C*

(*a*) Test for coenzyme I

Tubes contain: 1 ml. malic dehydrogenase preparation (*a*) from heart muscle, (*b*) from *coli*-juice.
1 ml. *M*/10 Na malate.
0·2 ml. 2*M* NaCN.
1 ml. phosphate buffer *p*H 7·2.
0·2 ml. 0·5 % methylene blue.

Additions	Reduction time	
	(*a*)	(*b*)
1 ml. water	>3 hr.	>3 hr.
1 ml. cozymase (=0·15 mg. coenzyme I)	1 min. 15 sec.	2 min. 25 sec.
1 ml. boiled material C (=20 mg. dry wt.)	>3 hr.	>3 hr.
1 ml. boiled organism (=20 mg. dry wt.)	20 min. 5 sec.	—

(*b*) Test for coenzyme II

Tubes contain: 1 ml. glucosemonophosphate dehydrogenase preparation.
1 ml. phosphate buffer *p*H 7·5.
0·3 ml. *M*/5 glucosemonophosphate.
0·2 ml. 0·5 % methylene blue.

Additions	Reduction time
1 ml. water	>2 hr.
1 ml. coenzyme II (=0·2 mg. coenzyme II)	15 min. 25 sec.
1 ml. boiled material C (=20 mg. dry wt.)	>2 hr.

Summary

1. The material obtained by grinding *Bact. coli* in the Booth-Green mill has been investigated for formic dehydrogenase activity.

2. The enzyme resides in the solid part of this material and has not been removed therefrom in an active state.

3. Digestion of this sediment with trypsin gives a material very active with methylene blue in the presence of formate but unable to utilize O_2 as H-acceptor.

4. Spectroscopic examination shows that the formic dehydrogenase of the sediment reacts normally with O_2 through cytochrome *b*. This is confirmed by the effects of cyanide, which inhibits the dehydrogenase in high concentrations.

5. Autolysis of the sediment yields a material which will oxidize formate with oxygen but which is quickly inactivated.

6. Inactivation of the enzyme occurs only when the dehydrogenase is incubated aerobically with formate.

7. The inactivation is not due to the production of oxalic acid, formaldehyde or hydrogen peroxide.

8. The inactivated enzyme can be reactivated by anaerobic incubation. This effect is accelerated by the presence of formate.

9. The inactivation is accelerated by increasing the O_2 tension. If the O_2 tension is decreased sufficiently, the inactivation is abolished.

10. The inactivation is accelerated by the action of cyanide on the carrier system (cytochrome) or checked by the addition of methylene blue.

11. The digested material will react with O_2 in the presence of methylene blue but the extent of the oxidation depends upon (*a*) the concentration of the dye and (*b*) the O_2 tension.

12. It is suggested that the enzyme reacts with O_2 along two paths: (1) directly leading to inactivation of the dehydrogenase activity, and (2) indirectly through the carrier system. The latter reaction, by diverting the course of the oxygen, protects the enzyme from the direct oxidation.

13. The digested material will give a steady O_2 uptake with a high concentration of cytochrome *c* and cytochrome oxidase in the presence of a low O_2 tension.

14. *Bact. coli* grown in air can oxidize formate linearly only if the O_2 tension is less than that of air.

15. A method for growing organisms on the surface of agar under any given O_2 tension is described.

16. Increased O_2 tension during growth leads to inhibition of formic dehydrogenase formation.

17. Formic dehydrogenase of *Bact. coli* does not require coenzymes I or II for its action.

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