LXIII. THE RELATIONSHIP BETWEEN THE RESPIRATORY CATALYSTS OF *B. COLI*.

BY ROBERT PERCIVAL COOK¹,
JOHN BURDON SANDERSON HALDANE
AND LESLIE WILLIAM MAPSON.

From the Biochemical Laboratory, Cambridge.

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DURING the process of respiration in the cell two different types of activation by catalysts have been described. On the one hand organic molecules are rendered more susceptible to oxidation, and on the other oxidising substances more prone to reduction. The first type of activation commonly leads to dehydrogenation in Wieland's sense, and is carried out by dehydrogenases. The activation of oxygen has been particularly stressed by Warburg [1926]. He describes the responsible catalyst as the *Atmungsferment*. As, however, we cannot agree with his view that it is the only catalyst concerned in respiration we prefer to employ the term oxygenase in accordance with the usual practice of naming an enzyme after its substrate. Bach's [1913] oxygenase is possibly not an oxygen activator, but an enzyme which, in conjunction with catechol or a related substance, reduces oxygen to hydrogen peroxide. The main object of the present research was to determine the relationship between these two types of activation. Whereas the dehydrogenations so far studied in living cells have usually been simple, the oxidations by molecular oxygen have almost always been complex. Thus the complete oxidation of glucose must involve very many consecutive reactions, and even that of alcohol or acetic acid more than one. Hence it is difficult to interpret the effects of heat, poisons, or other alteratives on such complex processes. Cook [1930] showed that after treatment with toluene-saturated water *B. coli* still performed the following oxidations:

The oxidation of formic acid proceeds to 98 % completion, those of succinic to fumaric, and of lactic to pyruvic acids proceed to about 82 % and 76 % respectively. In neither case does the oxidation proceed any further, no CO₂

¹ Beit Memorial Fellow.

being produced in the latter two. The rates do not differ greatly from those in the untreated organism.

The toluene-treated organism has the further advantage that it has no metabolism in the absence of added substrate. Not only does it not consume O_2 but it does not reduce methylene blue *in vacuo* even after several days.

The bacteria used were a strain (N.T.C.) from the National Type Culture collection, and a strain (O.P.S.) from the University Pathological Department, suspensions being made as described by Cook and Stephenson [1928], with frog Ringer's solution. These were diluted with water so as to give a suitable rate of reduction of oxygen or methylene blue. Fuller details are given by Cook and Alcock [1931]. Carbon monoxide was obtained from a cylinder. It contained small amounts of nitrogen and hydrogen as impurities, as well as CO_2 , which was absorbed in consequence of the technique used.

OBSERVATIONS ON THE DEHYDROGENASES OF THE TOLUENE-TREATED ORGANISM.

The properties of succinic, lactic, and formic dehydrogenases have been studied by Quastel and his colleagues from 1924 onwards and by Cook [1930], but certain further observations were required. The Thunberg vacuum tubes used contained 2 cc. 0.2 *M* phosphate buffer, 1 cc. 0.002 *M* methylene blue, 0.5 cc. 0.2 *M* substrate, and 1.5 cc. H_2O , to which inhibitors were added as required. These were brought to the required p_H with H_3PO_4 or NaOH.

Finally, 1 cc. of bacterial suspension diluted with water was added. The substrate concentration was thus *M*/120. The tubes were evacuated for 1 minute and then placed in a bath at 40°. The time taken for 90 % reduction was measured. All measurements were made at least in duplicate.

Effects of cyanide.

The effects of KCN on the rate of methylene blue reduction are shown in Fig. 1. The ordinates are the reciprocals of the reduction times. It will be seen that there is a moderate activation by low concentrations of cyanide, which is clearly significant in the case of succinic dehydrogenase. The greater effect of cyanide on formic dehydrogenase at the more acid p_H is doubtless due to the greater amount of HCN in equilibrium with cyanide ions at that p_H . The irregularities are perhaps due to the fact that variable amounts of HCN were pumped off in different experiments. It will be seen that there is no very serious inactivation of any of the dehydrogenases below a concentration of 0.002 *M*, whereas catalase and peroxidase are largely inactivated by concentrations of 10^{-5} *M*. Hence any marked effects of cyanide concentrations below 0.002 *M*, such as are shown in Fig. 3 are not due to effects on the dehydrogenases. No reduction of methylene blue occurs in presence of cyanide and buffer alone.

Effects of carbon monoxide.

In order to study the effect of CO this gas was passed through alkaline pyrogallol solutions to absorb O_2 and CO_2 . The substrate, dissolved in only 0.1 cc. of water, was placed in the small limb of the vacuum tube, which was evacuated; CO was run in, the tube re-evacuated and more CO added. It was then cooled on ice, shaken vigorously for exactly 3 minutes, and placed in a thermostat. Controls were filled in just the same way with N_2 . CO has no

Fig. 1. Effect of KCN on rates of methylene blue reduction.

effect on lactic dehydrogenase at 40° . At 25° it seems to have a small effect on formic dehydrogenase. Thus at 25° the reduction times in N_2 were 4.15, 4.25, 4.25, in CO 5.15, 5.45. However, at 16° there is no such effect. Thus reduction times in N_2 were 17.00, 17.10 and 16.45 minutes, in CO 16.40, 16.55 and 16.50. As the experiments on oxygen uptake in presence of CO were almost all made at this temperature, it is clear that the dehydrogenases were not being poisoned in them.

The Hecht-Eichholtz reagents and phenylurethane.

Hecht and Eichholtz [1929] and Eichholtz [1929] introduced the use of a system of organic compounds as heavy metal reagents in biochemical research. They form complexes with heavy metals, and the inhibition or acceleration [Krah, 1930] of a reaction is taken to show that the catalyst concerned contains a metal which combines with the reagent added. We have used the reagents 8-hydroxyquinolinesulphonic acid (Q), 2-aminophenol-4-sulphonic acid (A), and 1-amino-8-naphthol-4-sulphonic acid (N) at 40° . A has no marked effect on any of the dehydrogenases. Q has no effect on reduction by lactates. In very high concentrations such as 0.4 % Q slightly accelerates

reduction by succinate, the velocity being increased by 21 %. Q has a large effect on formate, as shown in Fig. 2. The effect is greater at p_H 6.3 than at 7.6, as with the acidic poisons used by Myrbäck [1926]. N has a very great inhibitory effect on reduction by formate. These results suggest that formic dehydrogenase contains copper and not iron. For Q and N, which inhibit, both

Fig. 2. Effect of 8-hydroxyquinolinesulphonic acid on rates of methylene blue reduction.

unite with copper. Q also unites with Mn, Co, and Ni, N also with Fe, while A unites with Fe only. Inhibition by Q is independent of variations in the concentration of methylene blue, and is no greater when the bacteria have stood with the reagent for $1\frac{1}{2}$ hours before the methylene blue is added.

No inhibition was obtained with phenylurethane on lactic or formic dehydrogenases.

EXPERIMENTS ON O_2 REDUCTION.

The Method.

Oxygen uptakes were studied in Barcroft apparatus. Each cup contained 1.0 cc. of a suspension of the toluene-treated organism, 1.0 cc. of a 0.5 *M* buffer solution, 0.5 cc. of a donator solution for which distilled water was substituted in the left-hand pot, and 0.5 cc. of distilled water or solution of an inhibitor. The p_H was always 7.6 in the case of succinate and lactate, this hydrogen ion concentration being nearly optimal, as shown by Cook and Alcock [1931]. In the case of formate, the p_H was either 7.6 or more usually the optimal 6.3. From time to time the p_H after the end of the reaction was measured with a hydrogen electrode and was found to be unaltered in the case of lactate and succinate. Formate solutions became more alkaline by about 0.2 p_H at most. The substrate concentration was usually *M*/60, as at this concentration the enzymes concerned are saturated with their substrates, and the rate of oxidation does not therefore fall off with a moderate diminution in the substrate concentration. Gas mixtures were made up and stored over water in aspirators. They were analysed with the Haldane apparatus.

To introduce them into the Barcroft apparatus the right-hand tube of the latter is connected to the aspirator with rubber tubing. The apparatus is then brought nearly into thermal equilibrium with the thermostat. The left-hand pot is left in position, the right-hand pot held so that a small crevice is left between it and its stopper. The gas is then introduced, the pot being held so close that there is always a positive pressure within it, as shown by the manometer. About half a litre is then run through, the pot pushed home and the tap closed. After 5 minutes' shaking in the bath the taps are opened to level the oil in the manometer, and the experiment begins. Analyses of gas from the pot agreed with that introduced to within 0.3 %. In a few experiments the manometers were evacuated, and both cups filled with gas mixture. On repeating the process several times the air is pretty completely replaced by gas mixture. The small central pot contained a roll of filter paper soaked in KOH to absorb CO_2 [Dixon and Elliott, 1930]. The apparent rates of oxygen uptake often did not become steady until half an hour had elapsed, due in part to the gases going into solution (see Fig. 5). The data here given refer to the linear parts of the curves. All experiments, including controls, were done at least in duplicate.

In the case of formate and lactate the oxygen uptake remained fairly constant from day to day. There were, however, occasional fluctuations, generally decreases in oxidising power on keeping. Hence we have not given full weight to experiments in which the effect of a poison was not measured simultaneously with a normal control, although when controls were done on the same day the error is likely to be small. In the case of succinate the oxidising power often fell off in the first few days of keeping a bacterial suspension. The power of reducing methylene blue sometimes fell off simultaneously; but often it remained normal when the oxygen uptake had dropped to less than 10 % of the original value. Hence our data on succinate oxidation are rather incomplete.

Effect of cyanide on oxygen uptake.

Precise measurements of the effect of cyanide on oxygen uptake are difficult, because the velocity of uptake, when inhibition is considerable, sometimes tends to fall off slightly with time. On the other hand, there is no question whatever as to the difference in the susceptibility of lactic dehydrogenase on the one hand, and succinic and formic on the other. This is at once clear from Fig. 3. The difference between the latter two is not so clearly significant, and may be due to the stimulating effect of cyanide on succinic dehydrogenase. As the dehydrogenases are hardly inhibited in concentrations below $10^{-3} M$ it is clear that the main effect here studied is a direct effect either on the oxygenase or on some intermediate substance. The dissociation constant of the oxygenase-cyanide compound (if we regard the effect as due to combination) would seem to be about $1.5 \times 10^{-3} M$ in the case of lactate, $5 \times 10^{-5} M$ in that of succinate, and $2 \times 10^{-5} M$ in that of formate.

Effect of phenylurethane on oxygen uptake.

Phenylurethane had a slight inhibitory effect on oxygen reduction by lactate and formate at 16°. The former process was reduced to 78 % of its original velocity by 0.5 cc. saturated phenylurethane solution in the total volume of 3 cc. Formate oxidation was reduced to 75 % by saturated phenylurethane solution and to 83 % by *M*/6000 solution.

Fig. 3. Effect of KCN on rates of O₂ reduction.

When, however, the oxidation had already been partly inhibited by cyanide, saturated phenylurethane was more effective. The uptake of lactate in presence of 10⁻³ *M* KCN was reduced to 24 % of its value with KCN alone, that of formate in presence of 10⁻⁵ *M* KCN to 56 % of its former value.

Effect of carbon monoxide.

A large number of experiments have been done in mixtures containing varying amounts of CO, O₂ and N₂. As a preliminary it was shown that the

Fig. 4. Oxygen uptakes at 16° and *p*_H 6.3 in presence of formate from air and two mixtures containing different amounts of CO, but similar CO/O₂ ratios.

oxygen uptake remains unaltered when air is replaced by pure O_2 , or diluted with N_2 so that the partial pressure of O_2 is reduced to 5 %. Hence neither diffusion of O_2 nor incomplete saturation of oxygenase with it was a limiting factor. Several experiments, of which Fig. 4 is typical, showed that as found by Warburg [1926] the inhibition by CO depends solely on the ratio of CO to O_2 , and not on the partial pressure of CO. It at once becomes clear that the oxidation of formate is more sensitive to CO than that of succinate or lactate. If V be the velocity of O_2 uptake in absence of CO, v that in its

Fig. 5. Oxygen uptakes at 16° and p_H 7.6 in presence of succinate from air and various gas mixtures containing CO.

Fig. 5 a. Data of Fig. 5 plotted after the first half-hour.

presence, r the ratio of partial pressures of CO to O_2 , and K the affinity of oxygenase for oxygen divided by its affinity for CO, then Warburg shows that $v = \frac{KV}{K+r}$, or $K = \frac{r}{\frac{v}{V} - 1}$, provided certain assumptions examined later are made.

From such data as those of Figs. 5, 6 and 7 it is possible to calculate values of K . Unfortunately the majority of our data are dubious for one of four reasons. Duplicates do not agree, uptakes are not linear, experiments with and without CO were not done simultaneously, or the temperature had varied. The results vitiated in any of these ways are grouped as second-class results in Table I. No results whatever have been rejected.

Details of a typical experiment are given below.

Two manometers containing N.T.C. bacilli and *M/60* formate at 16° and p_H 6.3 gave uptakes of 76.6 and 78.9 mm.³ O₂ in the half-hour between $\frac{1}{2}$ an hour and 1 hour after the beginning of the experiment. Two others containing the above in presence of 59.9 % CO and 7.49 % O₂ gave uptakes of 56.6 and 60.8 mm.³ O₂ in the 2 hours from $\frac{3}{4}$ hour to $2\frac{3}{4}$ hour after the beginning. In each case the O₂ uptake was linear over this period. The mean uptakes per hour were therefore 155.5 mm.³, and 29.34 mm.³. The velocity was thus reduced to 18.9 %, giving a *K* value of 1.86, with an uncertainty of ± 0.11 , allowing for the differences between duplicate oxygen uptakes and CO estimations.

Fig. 6. Oxygen uptakes at 16° and p_H 7.6 in presence of lactate from air and various mixtures containing CO.

Fig. 7. Oxygen uptakes at 16° and p_H 6.3 in presence of formate from air and various gas mixtures containing CO (O.P.S. bacilli).

On the other hand, in some of the second-class experiments duplicates disagreed very markedly. Thus in the lactate experiment giving a *K* value of 17.4 the duplicate CO uptakes were 21.1 and 27.7 mm.³. The lower value, which was probably correct, would have given $K = 11.1$.

It will be seen that the two strains used differed considerably as regards the sensitivity of formate oxidation to CO. There were no significant differences as regards succinate and lactate oxidation. The difference between the two strains possibly arose during the experiments. Two second-class experiments on the N.T.C. strain in July 1930 gave values of 4.15 and 3.20 for K , while the other experiments at p_H 6.3 from October onwards gave values of 2.35 or lower. There can be no serious doubts as to the significance of the differences of the K values found. It will be observed that there is no overlapping among the first-class values. The second-class values do not show any certain difference between succinate and lactate, but both these differ clearly from formate.

Table I.

Values of K (ratio of CO to O_2 giving 50 % inhibition) at 16°.

Substrate and race	First-class results	Median	Mean	Second-class results	Median
Succinate	5.9, 6.8	6.3	6.3	5.0, 5.0, 6.0, 10.3, 13.8, 16.5	8.2
Lactate	8.2, 9.5, 9.6, 9.7, 11.8, 12.2, 15.7*	9.7	10.1	3.8, 6.5, 6.9, 7.4, 7.8, 8.7, 10.2, 11.9, 13.4, 14.4, 16.4, 17.4	9.5
Formate (O.P.S.)	3.39, 3.45, 3.54, 4.14	3.49	3.62	1.75, 3.14, 3.45, 3.88, 4.46	3.45
Formate (N.T.C.)	1.86, 2.27, 2.35, 1.40†	2.27	2.15	0.94, 1.07*, 1.29, 1.67, 2.30, 2.95‡, 3.20, 4.15	1.98

* At 18°.

† At p_H 6.0.‡ At p_H 7.6.

In Fig. 8 the observed velocities for lactate oxidation in presence of CO, as percentages of those in its absence, are plotted against the CO/ O_2 ratio.

Fig. 8. Velocities of oxygen uptake (as percentages of that in air) at 16° and p_H 7.6 in presence of lactate from gas mixtures containing CO and O_2 in different ratios. The curve represents velocities calculated for $K=10$.

There is no decided evidence for a significant departure from a rectangular hyperbola, such as Warburg [1926] found in the case of yeast. The two velocities plotted on the right in Fig. 8 are certainly low, corresponding to low

values of K , but neither is reliable. On the other hand, in the case of formate oxidation by O.P.S. bacilli the two lowest velocities, obtained with CO/O_2 ratios of 13.6 and 13.9, give a first-class K of 3.39, and a second-class K of 3.20 respectively. Both the velocities lay between 18 % and 19 % of that in air.

At 40° the effect of CO was much less (Fig. 9). With formate we obtained a first-class value of 9.8, with the O.P.S. strain, and second-class values of

Fig. 9. Oxygen uptakes at 40° and p_{H} 6.3 in presence of formate from air and a gas mixture containing CO (O.P.S. bacilli).

Fig. 10. Oxygen uptakes at 16° and p_{H} 7.40 in presence of formate from a gas mixture containing 65 % CO and 7.7 % O_2 . After 45 minutes this was replaced by air.

40, 9.7, 6.7, and 5.7 with the N.T.C. strain. The effect on lactate oxidation was negligible with a CO/O_2 ratio of 13.2, which would have given 59 % inhibition at 16° . The value of K is probably at least 50.

Inhibition by CO is completely reversible (Fig. 10). Experiments carried out on bacteria exposed to CO in the strongest light at our disposal appeared to show an acceleration of about 5 % as compared with nearly complete darkness. The effect was, however, too small to measure accurately.

Combined effect of cyanide and carbon monoxide.

Four experiments were carried out to see what further inhibition was produced by CO on oxygen uptake already partly inhibited by cyanide. In two second-class experiments with lactate in presence of 0.001 *M* KCN we obtained values of 9.3 and 14.4 for *K*. With formate in presence of 0.001 *M* KCN we obtained, with N.T.C. bacilli, a first-class value of 2.16, and a second-class value of 2.47. Thus in presence of cyanide concentrations which themselves produce an inhibition of 37 % and 70 %, the value of *K* was, if anything, slightly increased, *i.e.* the system rendered less sensitive to CO.

Combined effect of oxalate and carbon monoxide.

As a preliminary to the use of oxalate it was shown that the values of *K* are unaltered by washing the bacilli free from calcium.

Oxalate inhibits the reduction of methylene blue by lactate, and has a similar effect on oxygen reduction. The latter is not, however, quite so great as the former. Thus in one series of experiments performed simultaneously the velocity of methylene blue reduction was reduced to 39.7 ± 2.3 %, that of oxygen reduction to 52.6 ± 1.9 %, the errors being the extreme values calculable from the observations.

Oxalate has no marked effect on O₂ reduction by formate [Cook, 1930] nor on the *K* value of this process. With N.T.C. bacilli we obtained a first-class value of 2.30 and a second-class value of 1.29 in 0.0027 *M* oxalate, in 0.0017 *M* oxalate a second-class value of 1.63. In 0.0076 *M* oxalate a first-class experiment gave *K* = 1.86. Thus, if anything, oxalate makes formate oxidation more sensitive to CO, but the effect, if any, is within the limits of experimental error.

On the other hand, oxalate renders the oxidation of lactic acid far less sensitive to CO. In a concentration of *M*/3000 we obtained a first-class value of *K* of 19.0, and second-class values of 42 and 468, which at least demonstrate that there was hardly any inhibition.

The combined effect of phenylurethane and carbon monoxide.

Although, as pointed out above, phenylurethane has only a slight effect on oxygen reduction, it renders the system extremely sensitive to CO. Thus the value of *K* for lactate oxidation in presence of 0.00011 *M* phenylurethane fell to 0.86 in one first-class experiment, that for formate oxidation by N.T.C. bacilli fell to 0.24 and 0.17 in two good second-class experiments. Clearly the ratio of the affinities of the oxygenase for CO and O₂ has been increased about tenfold. It will be remembered that phenylurethane also renders the system more sensitive to cyanide, though in this case the effect is less striking.

The effect of 8-hydroxyquinolinesulphonic acid.

In spite of its marked inhibitory effect on methylene blue reduction by formate, 8-hydroxyquinolinesulphonic acid has no effect on oxygen reduction by this substrate. Thus a 0.004 *M* concentration at 16° gave a 2 % reduction in oxygen uptake by formate, and a 1 % increase in that by lactate, both well within the limits of experimental errors. Nor is there any definite effect on the value of *K* in the case of formate. A second-class experiment on *N.T.C. bacilli* gave *K* = 1.68.

Experiments with mixtures of formate and lactate.

Cook [1930] showed that at 40° the oxygen uptake in presence of lactate and formate is equal to the sum of those in presence of either alone. This was found to be true at 16° also. Thus a suspension absorbed 64.3 mm.³ of O₂ in half an hour in presence of formate, 39.2 in presence of lactate, while in a mixture the quantity was 103.2 mm.³, the calculated value being 103.5. This mixture was now exposed to CO and gave a value of *K* of 1.66. The data are of the second-class, owing to disagreement of duplicates in presence of CO, and the true value was probably higher.

Oxygen uptakes in presence of methylene blue.

A series of experiments was carried out to see how far oxygenase can be replaced by methylene blue. Oxygen uptakes were measured alone, in presence of added methylene blue, of added cyanide and of methylene blue and cyanide. Fig. 11 shows that in the case of formate when the oxygenase has

Fig. 11. Oxygen uptakes from air at 16° and *p_H* 6.3 in presence of formate alone, with the addition of KCN, of methylene blue and of both.

been mainly poisoned by cyanide, it can be replaced to a considerable extent by methylene blue. This is reduced by the formic dehydrogenase and re-oxidised by air, this re-oxidation not being prevented by the cyanide. Fleisch

[1924] and Stephenson [1928] have obtained similar results. In other experiments the uptake in presence of formate has been actually increased by the addition of KCN and methylene blue. Thus at p_H 7.6 the oxygen consumption in the same period of half an hour was 47.0 mm.³ in the absence of cyanide and methylene blue, and 63.6 when both were present. Duplicates agreed well.

The oxidation of lactate was affected differently. We were never able to get more than 67 % of the original oxidation on adding methylene blue and cyanide, and methylene blue alone reduced the rate of uptake to about 74 % of the normal value. We have no satisfactory results in the case of succinate at 16°. At 40° methylene blue causes a slight acceleration.

The system in which, in presence of cyanide, oxygenase is replaced by methylene blue can be used as a model for the action of CO on the oxygenase. When such a system is exposed to air containing CO the rate of oxygen uptake falls off rather slowly, and finally may settle down to a definite velocity. In the steady state the methylene blue can be seen to be mainly reduced. This is explained by the discovery of Reid [1930] that the re-oxidation of leuco-methylene blue is a metal catalysis inhibited by CO. Presumably the KCN and CO between them unite with almost all the catalytically active metal. The degree of inhibition depends on the partial pressure of O₂ as well as that of CO. Thus in presence of formate at p_H 6.3 we obtained an uptake of 11.4 mm.³ per hour in presence of 52.5 % CO and 9.68 % O₂, but of 50.8 mm.³ in presence of 52.5 % CO and 46.3 % O₂. It had previously been shown that, in the absence of CO, the rate of oxygen uptake did not depend on the partial pressure of O₂. In another set of four experiments the rate was shown to be the same when the ratio of CO to O₂ was kept constant, the absolute amount being altered. The value of K was in one case 0.18, but it varied in different experiments, probably because the amount of catalytically active heavy metal was not constant.

DISCUSSION.

The experiments on methylene blue reduction can be interpreted without difficulty. The stimulating effect of small amounts of cyanide can probably be explained by its combination with small amounts of heavy metal which inhibit succinic dehydrogenase, as shown by Quastel and Wooldridge [1927]. A similar explanation of their activating effect on proteinases has been given by Krebs [1930]. The inhibition by relatively high concentrations of cyanide may be due to its union with heavy metals forming part of the enzymes. But the Hecht-Eichholz reagents give no indication of metal in succinic and lactic dehydrogenases, though they point strongly to the presence of copper in formic dehydrogenase and suggest that copper is inhibiting succinic dehydrogenase.

Cook [1930] showed, by the specific effect of malonate on the oxidation of succinate by methylene blue and oxygen, that the same catalyst was probably activating the succinate in each case, *i.e.* that succinate is activated by succino-dehydrogenase before it reduces oxygen. The same is true for lactate. But

this does not hold for formate, as 8-hydroxyquinolinesulphonic acid inhibits the reduction of methylene blue, but not of oxygen. And whereas methylene blue does not fully replace oxygenase in the oxidation of lactate, it more than does so in the case of formate. The suggestion is obvious either that oxygenase is the limiting factor throughout, or that the formate-activating mechanism which is inhibited by 8-hydroxyquinolinesulphonic acid is not normally concerned in oxygen reduction, but is made available for this process by the addition of methylene blue. The work of Stephenson and Stickland [1931] suggests that it may be related to hydrogenase in the same way as lactic dehydrogenase is related to oxygenase. The complexity of the curve relating oxygen reduction by formate to p_H [Cook and Alcock, 1931] suggests that the system concerned is particularly complex.

The fact that CO inhibits O_2 reduction in a competitive manner makes it quite clear that it acts on an activator of O_2 , as Warburg has pointed out. On the other hand, the difference between the apparent relative affinities of oxygenase in the three different oxidations studied suggests strongly that we are dealing with three oxygenases which differ from one another in this respect as do the haemoglobins of different species and individuals [Douglas, Haldane and Haldane, 1912; Anson *et al.*, 1924]. Further the relative affinities of the oxygenases concerned in formate oxidation in two different races of *B. coli* differ in this manner. Evidence for the existence of two oxygenases in *Galleria mellonella* which differ in their relative affinities has already been given by Haldane [1927].

It is clear, moreover, that, even if we do not accept the above theory, the actual molecules of oxygenase concerned in formate and lactate oxidation are different. For not only are the oxygen uptakes additive on mixing under normal conditions, but this is still true when they have been reduced by cyanide, so that oxygen activation is clearly the limiting factor. Thus in presence of $10^{-4} M$ KCN the oxygen uptake by formate was 70 mm.³ per hour, by lactate 80, by the two together 150. It therefore seems to be impossible that the three dehydrogenases should draw on the same common stock of oxygenase, even if the oxygenase associated with each is of the same molecular species.

Warburg [1926] believes that differences in the apparent affinity ratio K can be reconciled with the existence of only one oxygenase, on the following theory, re-stated in the terminology of Briggs and Haldane [1925] and Wurmser [1930]. Consider the reactions:

Where W represents oxygenase, and the molecular concentrations of the reactants and the velocity constants are as above. ϵk_3 is the constant of the

reaction actually measured and is supposed to be independent of the concentration of reducing substrate when this reaches a certain value, but to fall off when this is lowered, as in sugar-free buffer solution, ϵ being reduced from unity to a fraction.

Then the velocity of oxygen uptake is $\epsilon k_3 p$, or

$$\frac{\epsilon k_3 e x}{x + \frac{(k_2 + \epsilon k_3)}{k_1} \left(1 + \frac{k_4 y}{k_5}\right)}.$$

But x is very large compared with $\frac{k_2 + \epsilon k_3}{k_1}$, since if $y = 0$, the velocity is independent of x ; hence the velocity is

$$\frac{\epsilon k_3 e}{1 + \frac{(k_2 + \epsilon k_3) k_4 y}{k_1 k_5 x}}.$$

Hence $K = \frac{k_1 k_5}{(k_2 + \epsilon k_3) k_4}$, and therefore lies between $\frac{k_1 k_5}{(k_2 + k_3) k_4}$ and $\frac{k_1 k_5}{k_2 k_4}$.

If k_2 is small compared with k_3 , K may become very large when ϵ is small. The fact that the value of K for lactate oxidation rose when the lactic dehydrogenase was inhibited by oxalate completely confirms Warburg's theory. But it is also clear that the value of K can be altered by other means. For phenylurethane does not greatly reduce the velocity of oxygen uptake in the absence of CO, and hence cannot greatly change ϵ . But yet it has a very large effect in lowering the value of K .

On the theory that there is only one oxygenase, or *Atmungsferment*, we should have to suppose that whereas most of the oxygenase concerned in formate oxidation is in use, only a fraction is active in the case of succinate, and a still smaller fraction in that of lactate. In this case, however, KCN, by inhibiting part of the oxygenase, should diminish the value of K . It does not. The order of susceptibilities to KCN is the same as that to CO, but in this case the apparent affinities are nearly the same for formate and succinate oxidation, both being very much greater than for lactate oxidation. In the case of CO formate is far more susceptible than succinate or lactate. Moreover, whereas the oxygenases associated with lactate and formate oxidation are very stable, that concerned with succinate is unstable, even when the dehydrogenase is unaltered. Again it is known that the oxygenases of different organisms differ widely in the susceptibilities of their CO compounds to light [Keilin, 1929] and that this difference does not run parallel with their apparent affinities to CO, as it should if ϵ is the only variable concerned. Hence without ruling out the possibility of an explanation on Warburg's lines, we regard it as probable that there are at least three different kinds of oxygenase in *B. coli*. They probably all possess the same prosthetic group, but differ, as do haemoglobins, in the molecule to which it is attached.

We find that the inhibition by CO falls off very strongly with temperature. Warburg [1926] found no definite temperature effect, and Keilin [1929] found that in the case of yeast a rise of temperature increased the inhibitory effect of CO on oxygen consumption while having no effect on CO inhibition of

indophenol oxidase. On the other hand, the inhibition by CO of oxygen uptake by heart muscle and potato catechol oxidases showed a falling off with rising temperature, as in our case. We believe that this is due to a real diminution in the ratio of the affinities for CO and O₂, because there is a strong reason to think that oxygen activation is a limiting factor at 40°, even if it is not so at 16° [Cook, 1930]. Thus the majority of cases show a rise of *K* with temperature. The corresponding quantity for a human haemoglobin rises from 0.0025 at 15° to 0.004 at 37° [Anson *et al.*, 1924]. (Warburg's *K* is the reciprocal of Barcroft's.)

Finally we have to consider the relation between a dehydrogenase and the oxygenase associated with it. Three possibilities are open. Intermediate "carriers," such as cytochrome, glutathione, or hexuronic acid may diffuse from one to the other more or less freely. One or more molecules of each may be associated in a very small section of the cell, a minute reaction vessel, so to speak. Or the dehydrogenase and oxygenase molecules may be actually in contact. Our results are strongly against the first hypothesis, for we have shown that there is not a common stock of oxygenase, and on the whole in favour of the third. If it is true we can expect both oxygenase and dehydrogenase activity to be limiting factors simultaneously, as, for example, p_H and substrate concentration are in the case of a simple enzyme. Otherwise it is difficult to see why at 16° both CO and oxalate should give approximately hyperbolic curves when inhibiting lactate oxidation. If there were, for example, an excess of oxygenase, we should expect to get no effect from CO until a large amount of the oxygenase was poisoned. But we obtained 25.6 % inhibition of lactate oxidation with a ratio of CO to O₂ of only 2.4, the calculated inhibition being 19 %. For this reason an actual juxtaposition of the two catalysts seems plausible, though far from certain. Case [1931] comes to a similar conclusion regarding two muscle enzymes.

The results obtained must not be regarded as a picture of the mechanism of respiration in a typical cell. *B. coli* is a facultative anaerobe, its dehydrogenases are almost if not quite confined to its surface, and Dr Keilin kindly allows us to state that he finds it to contain cytochrome B and protohaematin, but has been unable to detect cytochrome A or C. A more normal type of cell might behave quite differently.

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SUMMARY.

1. We have studied the oxidation of succinate to fumarate, lactate to pyruvate, and formate to bicarbonate by the toluene-treated *Bacillus coli*. These reactions can each be studied uncomplicated by others.

2. Oxidation by methylene blue is little affected by KCN or CO, but the effects of other poisons suggest that formic dehydrogenase contains copper.

3. Oxidation by O_2 is inhibited by KCN and CO. The activators of oxygen concerned in the three catalyses differ in their susceptibilities to these poisons, and are regarded as differing as do different haemoglobins.

4. Phenylurethane has little direct action on these catalysts, but makes them more sensitive to CO and KCN.

5. Conclusions are drawn as to the way in which the various catalysts which are concerned in respiration co-operate with one another.

6. In presence of cyanide methylene blue can replace the activators of oxygen. Like theirs, its action is inhibited by CO.

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