CLXVIII. THE REDUCTION OF NITRATES BY *BACT. COLI.*

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Introduction.

It has long been known that many bacteria are able to reduce nitrates to nitrites, and this fact is extensively used in classification. More exact investigation of the process is of recent date. Quastel, Stephenson and Whetham [1925] showed that nitrate could produce anaerobic growth of some facultative anaerobes in media which without it would not support growth, *i.e.* the nitrate could take the place of molecular oxygen. The coupled reaction, with reduction of nitrate to nitrite, always yields less energy than the corresponding direct oxidation, *e.g.*,

$$\text{lactic acid} + KNO_3 \rightarrow \text{pyruvic acid} + H_2O + KNO_2 + 30{\cdot}7 \text{ cals.}$$

$$\text{lactic acid} + \tfrac{1}{2}O_2 \rightarrow \text{pyruvic acid} + H_2O + 51{\cdot}9 \text{ cals.}$$

Quastel and Whetham [1924] showed that the utilisation of nitrate as a hydrogen acceptor depended on the power of the organism to activate the nitrate molecule, by demonstrating that nitrates oxidise leucomethylene blue in the presence of a washed suspension of cells but not in its absence. The capacity of a species of bacteria for anaerobic growth on, say, lactate and nitrate, is, of course, parallel to its power of oxidising leucomethylene blue by nitrate.

In its rôle as a supporter of growth in media containing only non-fermentable carbon compounds nitrate is strictly comparable with molecular oxygen, and the object of the work described here is to make a comparison between the reduction of nitrate to nitrite and that of oxygen to water.

In considering the enzymic mechanisms involved, there appear to be three possibilities, *viz.*,

(1) the activations of oxygen and nitrate are carried out by the same enzyme;

(2) the enzymes concerned are different, and specific for oxygen and nitrate respectively;

(3) the activation of nitrate is carried out by an accessory mechanism in

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close connection with the oxygen-activating enzyme. This last is a very vague conception, which may have to be considered if the other hypotheses fail.

Methods.

The organism chosen was *Bact. coli* (Escherich); two different preparations of the cells were used, first an ordinary washed and aerated suspension, and second the same suspension treated with toluene at room temperature [Quastel and Wooldridge, 1927; Cook, 1930]. For different purposes the one or the other proved more convenient, but wherever the same experiment could be carried out with both, essentially similar results were obtained. The chief differences of behaviour produced by toluene treatment are (1) the endogenous respiration of the cells is reduced to negligible proportions, *i.e.* methylene blue, oxygen and nitrate are not reduced except in the presence of an added donator such as hydrogen, formate, lactate, *etc.*, (2) the oxidation of lactate and succinate proceeds only as far as pyruvate and fumarate respectively, and (3) the suspension is incapable of attacking glucose.

The course of the reduction of nitrate was followed by two methods. The first consisted of placing the cell suspension in the cups of a Barcroft differential manometer, with a measured amount of nitrate in the right-hand cup, filling the apparatus with hydrogen, and observing the rate at which hydrogen was absorbed [*cf.* Stephenson and Stickland, 1931]. The other method was carried out by keeping the suspension anaerobic in the presence of a known concentration of nitrate and of hydrogen donator (*e.g.* lactate) and estimating the nitrite produced, at various time intervals, colorimetrically by means of the Griess-Ilosvay reagent. The most convenient apparatus for this purpose was the ordinary Thunberg tube. A set of tubes containing nitrate, donator (usually lactate), buffer and cell suspension was evacuated and placed in a bath, and for each reading one tube was taken out and the concentration of nitrite in it estimated.

To prepare the reagent completely colourless the sulphanilic acid and α-naphthylamine were recrystallised from water and light petroleum respectively, dissolved in 50 % acetic acid made with glass-distilled water, and mixed in equal proportions when required. Glass-distilled water was used in making up all solutions, as the ordinary distilled water often contained appreciable amounts of nitrite. The appropriate quantity of the solution to be estimated was added to 5 cc. of the reagent, and made up to 20 cc. with 50 % acetic acid. The standard was a 0·0001 *M* solution of pure recrystallised silver nitrite, of which 1 or 2 cc. were used. The maximum colour developed in 15–20 minutes and was stable for at least 24 hours. By this method duplicate tubes always agreed to within 3 %. All the experiments were carried out at 40° in the presence of phosphate buffer at p_H 7·0, except where otherwise stated.

Preliminary observations.

(1) *The extent of reduction of nitrate.* With toluene-treated cells, using the Barcroft manometer method, the hydrogen taken up corresponded to 90–100 % of the theoretical for reduction to nitrite, *e.g.*

	Amount of KNO_3	H_2 uptake mm.³	Theoretical	%
1	0·5 cc. *M*/50	227	224	101
2	,,	210	224	94
3	,,	215	224	96

Other donators, added in excess to known amounts of nitrate in Thunberg tubes, with either normal or toluene-treated suspensions, also gave 100 % reduction as measured by nitrite estimations, *e.g.* with lactate,

	KNO_3 added *M*	Nitrite found *M*	%
1	0·0250	0·0245	97·5
2	0·0200	0·0197	98·5
3	0·0200	0·0198	99

With untreated suspensions, the hydrogen uptake by the first method always fell short of the theoretical, owing to reduction of part of the nitrate by other donators in the cells [see Stephenson and Stickland, 1931], but estimation of nitrite showed the reduction to be complete.

(2) *The extent of oxidation of hydrogen donators.* Cook and Stephenson [1928] showed that with oxygen as the hydrogen acceptor an untreated suspension of *Bact. coli* oxidised formate completely, while with lactate the oxygen uptake was two-thirds of that required for complete oxidation, and with succinate there was a rapid oxidation as far as fumarate with an indefinite and slower further oxidation. It has been impossible to get parallel results with nitrate, owing to the great rapidity with which the untreated cells reduce nitrate in the absence of added donators.

In the case of toluene-treated suspensions, Quastel and Wooldridge [1927] and Cook [1930] have shown that formate is completely oxidised in oxygen,

Donator	Conc. of donator *M*	Conc. of NO_2^- *M*	Extent of oxidation %	Average %	O_2 uptake (Cook) %
Lactate	0·004	0·00332	83		
		0·00353	88		
		0·00317	79	82	78
	0·002	0·00159	80		
		0·00162	81		
Succinate	0·004	0·00312	78		
		0·00337	84		
		0·00303	76	82	82
	0·002	0·00168	84		
		0·00178	89		
Formate	0·0051	0·00472	93		
		0·00461	90		
		0·00472	93	92	98
		0·00465	91		
	0·00255	0·00238	93		
		0·00231	91		

while lactate and succinate go as far as pyruvate and fumarate respectively. The results for reduction of nitrate were obtained by incubating tubes containing a measured amount of the donator and excess of nitrate until there was no further increase in the nitrite concentration. The results are given above, with Cook's [1930] figures for the percentage of the theoretical oxygen uptake in the last column. The oxidations are incomplete, but proceed as far as they do with oxygen.

(3) *The linearity of the reaction.* Starting with 0·02 *M* potassium nitrate and 0·02 *M* lactate, and using a dilute suspension of cells, the initial course of the reaction may be determined while less than 1 % of the reaction is taking place, and the rate was found to be constant within the limits of accuracy of the method (Fig. 1). With a thicker suspension the whole course of the reaction may be followed, as in Fig. 2.

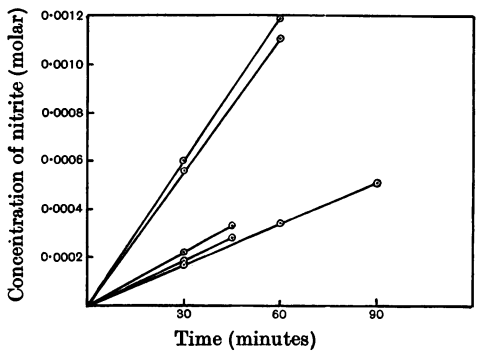

Fig. 1.

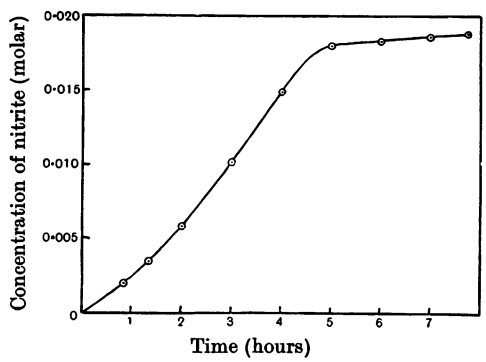

Fig. 2.

(4) *The affinity of the enzyme for nitrate.* The initial velocity of the reaction was measured at a series of nitrate concentrations by the vacuum tube method. No great decrease in velocity with diminishing concentration was observed until concentrations of nitrate were reached at which the method of estimation

of the nitrite had reached its limit, so the lowest points on the curve (Fig. 3) are not very accurate; it was possible, however, to make an estimate of the

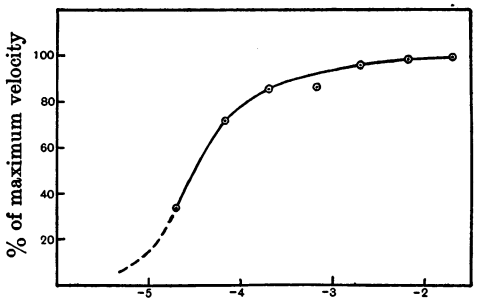

Fig. 3.

affinity, and the three separate values obtained for the Michaelis constant were 0·00005, 0·00003 and 0·00005 *M*. Hence one can be sure of being able to get initial velocity values at concentrations of nitrate down to at least 0·0005 *M*.

(5) *The effect of* p_H *on the velocity.* Using the vacuum tube method for measuring the rates, the curves shown in Fig. 4 were obtained, giving the

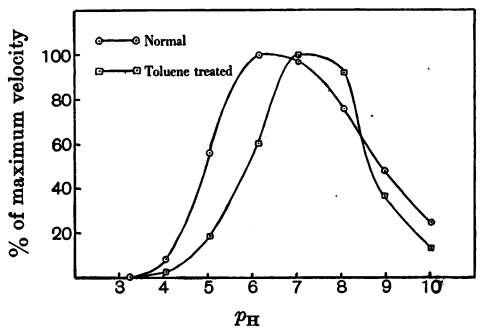

Fig. 4.

relation between p_H and initial velocity of nitrate reduction. Treatment with toluene causes a marked change in the p_H curve. Both curves are given as percentages of the maximum velocity, but the actual optimum with the toluene-treated suspension is only about 60–70 % of that before treatment, so that the apparent acceleration at p_H 8 is not real.

(6) It was also shown that variation of the lactate concentration within narrow limits about 0·02 *M* caused no great difference in the reaction velocity:

Lactate conc. (*M*)	Initial velocity (*M* NO_2^- per hour)
0·02	0·00081
0·0067	0·00079
0·002	0·00069

As a consequence of these preliminary results, the following was used as a standard method.

1 cc. 0·1 *M* potassium nitrate, 1 cc. 0·1 *M* sodium lactate, 1 cc. phosphate buffer p_H 7·0, 1 cc. water and 1 cc. of the suspension of cells were placed in each Thunberg tube, and the tubes were evacuated and placed in a water-bath at 40°. At 30 and 60 minute intervals tubes were removed and the nitrite in them was estimated, the concentration of cells being adjusted so that after 30 minutes roughly 1 % of the nitrate was reduced. The two points almost always lay on a straight line through the origin, and experiments showing any significant departure from linearity were discarded.

The effect of cyanide on nitrate reduction.

Most biological oxidations involving the use of molecular oxygen are strongly inhibited (though not always completely) by low concentrations of cyanide of the order of 0·0001–0·001 *M*. In the case of *Bact. coli* it has been shown [Cook, Haldane and Mapson, 1931] that the inhibition of the oxidation of formate, lactate and succinate tends towards 100 % as the cyanide concentration is increased.

The inhibitory effect of cyanide on nitrate activation may be shown qualitatively in the following simple way. Four sets of vacuum tubes containing the following solutions

	Buffer cc.	Methylene blue cc.	KNO_3 *M*/40 cc.	KCN *M*/200 cc.	Water cc.	Bact. susp. cc.	Reduction time at 40° min.
1	1	1	—	—	2	1	7·75
2	1	1	1	—	1	1	∞
3	1	1	—	1	1	1	5·25
4	1	1	1	1	—	1	8

were evacuated and filled with hydrogen, with the usual precautions for preventing loss of hydrogen cyanide. The reduction times show that whilst nitrate prevents the reduction of methylene blue, this inhibition is almost completely removed by cyanide.

Quantitatively each of the two methods described has been applied to the measurement of the inhibition, with similar results. With the Barcroft manometer method special precautions must be taken to prevent loss of hydrogen cyanide, either in filling the cups with hydrogen or by absorption in the potash which usually occupies the small tubes inside the cups. The former difficulty was overcome by thoroughly evacuating the solutions used before placing them in the apparatus, to remove dissolved oxygen, and then partially evacuating and filling with hydrogen several times until the amount of air left was negligible; the latter by omitting the potash from the cups. This was justified by the fact that no carbon dioxide can be produced in the reaction and also by direct comparison of experiments with and without potash. In this way the velocity of hydrogen uptake at various concentrations of potassium cyanide was compared with that in the absence of cyanide.

The results with lactate as donator were obtained by using vacuum tubes with hollow stoppers to contain the cyanide solution, which was mixed with the rest of the contents of the tube after evacuation; the alkalinity of the potassium cyanide solution was sufficient to prevent appreciable loss of the acid during evacuation.

The results of all the measurements are given in Fig. 5. Toluene treatment

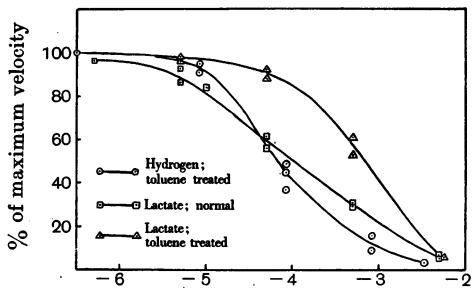

Fig. 5.

of the suspension has a considerable effect on the inhibition of nitrate reduction by cyanide, making the enzyme more susceptible to the reagent.

The extent of the inhibition does not depend on the nitrate concentration, *i.e.* it is as usual non-competitive.

KCN *M*	KNO_3 *M*	Velocity (*M* NO_2^-/hr.)	Velocity of control	Inhibition %
0·00002	0·02	0·000043	0·000114	62
0·00002	0·001	0·000035	0·000101	65

The effect of carbon monoxide.

The Barcroft manometer method of measuring the velocity of reduction cannot be applied in this case, as carbon monoxide has an inhibiting effect on hydrogenase which would interfere; the following figures show the effect of about half an atmosphere pressure of carbon monoxide on the rate of reduction of methylene blue by hydrogen in vacuum tubes with a toluene-treated suspension of *Bact. coli*:

Hydrogen mm.	Carbon monoxide mm.	Reduction time (mins.)
200	0	7·5
180	325	18·75

The lactic dehydrogenase is entirely unaffected by carbon monoxide [Cook, Haldane and Mapson, 1931], so the vacuum tube method can be applied; this is one of the reasons why lactate was chosen as the standard donator in this work.

Wherever carbon monoxide inhibits respiration, the inhibition is found to be a competitive one, so if the analogy between oxygen respiration and nitrate

respiration is to be borne out the inhibition by carbon monoxide should be greatest at the lowest nitrate concentrations. Neither at high or low nitrate concentrations was any inhibition observed, *e.g.*

KNO_3 (M)	CO (mm.)	Velocity (M NO_2^-/hr.)
0·02	0	0·000431
	117	0·000438
	325	0·000433
0·002	0	0·000384
	120	0·000410
	315	0·000391
0·0002	0	0·0000270
	650	0·0000265

It is essential in these measurements to find the initial velocity, as any change in the nitrate concentration would produce a double error, partly due to the assumed increase in inhibition by carbon monoxide at the lower nitrate concentration, and partly to the fact that the enzyme is not saturated with its substrate at 0·0002 M, so that the velocity would fall off as the concentration decreased. The lowest nitrite concentration that can be estimated with reasonable accuracy by the method employed here is 0·00001 M, so that if anything like an initial velocity is to be measured the initial concentration of nitrate must not be taken below, say, 0·00004 M.

Using this minimum concentration of nitrate, several experiments have been carried out. 3 cc. of each of 0·0002 M potassium nitrate, 0·1 M sodium lactate, phosphate buffer at p_H 7·0 and water were measured into each of six 100 cc. Büchner flasks, and 3 cc. of cell suspension added at such a dilution that about 0·00001 M nitrite would be expected in 30 minutes. The flasks were evacuated and one-half of them filled with carbon monoxide purified from oxygen. After 30 minutes' immersion in the water-bath, with vigorous shaking at first to ensure saturation with the gas, they were taken out and 10 cc. of their contents used for nitrite estimations. Typical results were

		Velocity (M NO_2^-/hr.)
1	Without CO	0·0000164
	With CO (about 1 atmosphere)	0·0000160
2	Without CO	0·0000212
	With CO (about 1 atmosphere)	0·0000203

These differences are not outside the experimental error, so the conclusion is that at a concentration of nitrate of 0·00004 M one atmosphere pressure of carbon monoxide (about 0·0007 M CO in solution) produces no measurable inhibition.

The effect of oxygen.

Before this subject could be investigated, a few preliminary results were required. First, it was shown that *Bact. coli* cannot re-oxidise nitrite to nitrate by means of oxygen. Tubes containing buffered 0·00025 M sodium nitrite and a thick cell suspension were aerated with a current of air saturated with water vapour, and samples were withdrawn for estimation of nitrite. No re-oxidation was observed.

Time (mins.)	$NaNO_2$ (M)
0	0·000246
30	0·000248
60	0·000260
140	0·000247

Second, the rates of reduction of nitrate in the absence of oxygen and of oxygen in the absence of nitrate were compared directly. It was found that, taking one atom of oxygen as comparable with one molecule of nitrate, oxygen is reduced about ten times as fast as nitrate, *e.g.*

Normal suspension	0·0221 g. atoms O_2 per hour 0·00236 g. mols. KNO_3 per hour
Toluene-treated suspension	0·0034 g. atoms O_2 per hour 0·000352 g. mols. KNO_3 per hour

To investigate the inhibition of nitrate reduction by oxygen quantitatively, it was necessary to vary the concentration of oxygen used, and this was done by diluting air with nitrogen and bubbling the mixtures through the solutions. The details of the method employed are as follows. A large aspirator was filled with the gases in roughly the right proportions, and the oxygen in the mixture estimated. 2 cc. of 0·1 M sodium lactate, phosphate buffer at p_H 7·0, water and nitrate solution were measured into each of a series of large test-tubes with corks, connected with pressure tubing, and a rapid stream of the gas bubbled through for about 15 minutes to saturate the solutions with oxygen at the right pressure. Then 2 cc. of the suspension of bacteria were added, and the bubbling was continued while samples were withdrawn for estimation of nitrate at various intervals. The velocity of nitrate reduction *in vacuo* was determined simultaneously. To make sure that at the lowest oxygen pressures no appreciable part of the oxygen was being removed from the gas, a sample was collected in one experiment as it issued from the last tube, and analysed. Before passing through the tubes the gas gave duplicate analyses of 0·42 % and 0·41 % oxygen, and after, 0·41 % and 0·44 %.

A series of results was obtained with nitrate concentrations of 0·02 M and 0·002 M; the extent of the inhibition was identical in the two cases, so only one set of figures (those for 0·02 M) is given. These results are plotted in Fig. 6, the concentrations of oxygen being given logarithmically.

O_2 %	O_2 in sol. M	Velocity	Velocity of control	Inhibition %
99	0·00100	0·000046	0·00120	96
20·9	0·00021	0·000026	0·000513	94
3·76	0·000038	0·000026	0·000387	93
1·65	0·000017	0·000047	0·000412	88
1·09	0·000011	0·000136	0·000352	61
0·42	0·0000042	0·000259	0·000350	26
0·36	0·0000036	0·000446	0·000568	21

For reasons which will be explained in the discussion of these results, the effect of carbon monoxide on the inhibition of nitrate reduction by oxygen was investigated. Experiments similar to those already described were done

with mixtures containing different amounts of oxygen with roughly ten times as much carbon monoxide. The inhibition was in each case much decreased (see Fig. 6).

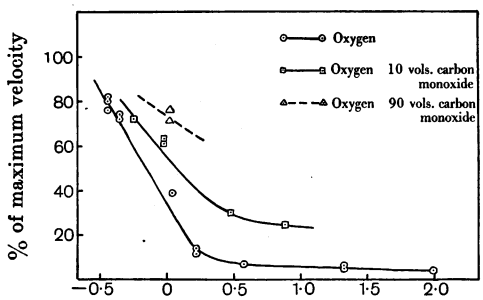

Fig. 6.

O_2 %	CO %	CO/O_2	O_2 in sol. *M*	CO in sol. *M*	Velocity	Velocity of control	Inhibition %
7·62	84·3	11·1	0·000077	0·00063	0·000124	0·000508	75·5
3·00	31·9	10·6	0·000030	0·00024	0·000132	0·000440	70
0·94	9·8	10·5	0·0000095	0·000073	0·000227	0·000370	39
0·55	4·5	8·9	0·0000056	0·000033	0·000398	0·000528	24
In addition one experiment was done with a larger CO/O_2 ratio:							
1·03	91·2	89	0·0000104	0·00068	0·000296	0·000391	24

A further question of importance is whether the extent of inhibition by oxygen depends on the concentration of nitrate. As already stated, initial concentrations of nitrate of 0·02 *M* and 0·002 *M* gave identical results, in the presence or absence of carbon monoxide, at all concentrations of oxygen between 0·000004 *M* and 0·001 *M* (0·4 % and 99 % of an atmosphere). The following results, obtained in the same way, show that the inhibition is also the same with 0·0002 *M* nitrate, a concentration at which the velocity of reduction is already falling off owing to unsaturation of the enzyme.

O_2 %	O_2 in sol. *M*	KNO_3 *M*	Velocity	Velocity of control	Inhibition %
0·36	0·0000036	0·02	0·000446	0·000568	21
,,	,,	0·002	0·000416	0·000547	24
,,	,,	0·0002	0·000332	0·000402	18

A few experiments were carried out to see if nitrate affects the velocity of reduction of oxygen. Two pairs of Barcroft manometers were set up, one pair with 0·02 *M* sodium lactate, the other with 0·02 *M* lactate and 0·01 *M* nitrate, both in air. The nitrate seemed to slow down the oxygen uptake about 5 %, but this is not really outside the experimental error, and cannot be considered significant.

Discussion.

There is no difficulty in deciding between the three possibilities mentioned in the introduction, with regard to the enzymes concerned in the activation of oxygen and of nitrate.

First, the relative affinities of the enzymes for their substrates and for carbon monoxide show that two entirely different mechanisms are concerned. Cook, Haldane and Mapson [1931] state that oxidations by oxygen are still working at their maximum velocity in oxygen at 4 % of an atmosphere pressure, *i.e.* at 0·00004 *M*, so that the Michaelis constant of the oxygen enzyme for its substrate is at any rate less than 0·000004 *M*; and they showed that the affinity of the enzyme for carbon monoxide is one-tenth that of its affinity for oxygen (when lactate is the hydrogen donator), so that the Michaelis constant of carbon monoxide for the oxygen enzyme is at any rate less than 0·00004 *M*. In the case of nitrate, the Michaelis constant for the substrate is about 0·00005 *M*, while carbon monoxide (0·0007 *M*) causes no inhibition in the presence of 0·00004 *M* nitrate, so that the Michaelis constant of the nitrate enzyme for carbon monoxide must be at any rate greater than 0·007 *M*. The values $< 0{\cdot}00004$ and $> 0{\cdot}007$ are sufficiently divergent to show that the two enzymes cannot be the same.

Second, if the same enzyme activated both oxygen and nitrate, the inhibition of the reduction of one substrate caused by the presence of the other should be competitive, whereas the results show that the amount of inhibition at any oxygen pressure is independent of the nitrate concentration. Thus the facts can be accounted for only by assuming two separate specific enzymes.

There remains to be explained the fact that oxygen causes a non-competitive inhibition of nitrate reduction, and a simple and qualitatively satisfactory explanation will now be given. Lactic dehydrogenase is providing a constant supply of activated lactic acid, and this, or the intracellular hydrogen carriers reduced by it, is shared between the oxygen and the nitrate enzymes. When both these enzymes are saturated with their substrates, the relative amounts reduced will depend on the "concentrations" of the enzymes and on various other factors. Considering the facts that oxygen is reduced ten times as fast as nitrate, and that the maximum inhibition of nitrate reduction by oxygen is 96 %, it will be seen that under these conditions 0·4 % of the available lactate is oxidised by nitrate and 99·6 % by oxygen. If now the rate of activation of oxygen is decreased by lowering the oxygen pressure, a greater proportion of the lactate will be oxidised at the nitrate enzyme; *e.g.* at 0·36 % oxygen, giving 20 % inhibition of the nitrate reduction, 8 % of the available lactate is oxidised by nitrate, 92 % by oxygen. Similarly, if the activation of oxygen is inhibited by carbon monoxide, a greater proportion of nitrate will be reduced; a CO/O_2 ratio of ten, giving 50 % inhibition of oxygen uptake, reduced the limiting inhibition from 96 % to about 80 %, so that 2 % of the lactate was oxidised by nitrate and 98 % by oxygen. It can be seen that nitrate cannot alter the rate of oxygen uptake sufficiently to give significant results on a Barcroft manometer apparatus.

Though the system is too complicated for exact mathematical treatment, the facts are qualitatively explained by this view.

In its behaviour with cyanide the nitrate-activating enzyme is similar to

other oxidising enzymes such as indophenol oxidase and peroxidase; with *Bact. coli*, using lactate as hydrogen donator, 50 % inhibition of the oxygen-activating enzyme is produced by 0·001 *M* cyanide, and 50 % inhibition of the nitrate enzyme by 0·0001 *M*. The inhibition is non-competitive in both cases [Cook, Haldane and Mapson, 1931]. The lack of inhibition by carbon monoxide is not unusual; Dixon [1928] has shown that the catalysis of the oxidation of cysteine by haematin is not inhibited by this reagent.

Summary.

1. *Bact. coli* reduces nitrate quantitatively to nitrite. After toluene treatment it oxidises formate, lactate and succinate to the same degree by means of nitrate as it does with oxygen, *i.e.* to carbon dioxide, pyruvate and fumarate respectively.

2. The reduction can be completely inhibited by cyanide, 50 % inhibition being produced by about 0·0001 *M* KCN.

3. As far as the method used allowed, no inhibition by carbon monoxide could be observed.

4. Oxygen causes a non-competitive inhibition of nitrate reduction; this inhibition is partly removed by carbon monoxide.

5. A theory is offered which explains these results qualitatively.

I wish to record my thanks to Miss Stephenson, Drs Dixon and Woolf, and Prof. J. B. S. Haldane for useful advice, and to Sir F. G. Hopkins for much help and encouragement.

REFERENCES.

Cook (1930). *Biochem. J.* **24**, 1538.
—— Haldane and Mapson (1931). *Biochem. J.* **25**, 534.
—— and Stephenson (1928). *Biochem. J.* **22**, 1368.
Dixon (1928). *Biochem. J.* **22**, 902.
Quastel, Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
—— and Whetham (1924). *Biochem. J.* **18**, 519.
—— and Wooldridge (1927). *Biochem. J.* **21**, 148.
Stephenson and Stickland (1931). *Biochem. J.* **25**, 205.