CLXIX. A COMPARISON OF THE DEHYDROGENATIONS PRODUCED BY *B. COLI COMMUNIS* IN THE PRESENCE OF OXYGEN AND METHYLENE BLUE.

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THE chemical changes brought about by bacteria may be studied in two ways. The first and more general method is to study the chemical changes in a medium of known composition where active growth is taking place. The second method is to act on a given substrate with relatively large amounts of already grown bacteria under conditions where growth is known not to occur. This method has been used by Quastel and his collaborators [1924-1928] with great success. The bacteria are termed "resting" or non-proliferating bacteria. Quastel [1928] has reviewed the method and its results.

The interest of this work lies in the fact that using such systems we may study the enzymic reactions of bacteria uncomplicated by the synthetic growth processes. That the processes we are studying may be correlated with the general problem of bacterial growth appears probable from the experiments of Quastel, Stephenson and Whetham [1925], and Quastel and Stephenson [1925]. It must be pointed out, however, that with the washed bacterial cells we are dealing with very complex enzyme systems bound up in the cell. Quastel and Wooldridge [1927, 2] have shown that the enzymic changes brought about by the resting bacteria are due rather to the fact that bacteria possess the enzyme systems concerned than to the fact that they form organised systems. The action of various growth inhibitors on resting bacteria shows that while certain mechanisms can be eliminated others are left intact.

The work to be described here consists of a study of the dehydrogenations produced by normal resting bacteria and bacteria that have been treated with various growth inhibitors. The dehydrogenations were performed in the presence of various hydrogen acceptors. Methylene blue and oxygen were particularly studied, as a comparison of the data gives interesting results on the anaerobic and aerobic oxidative processes of the bacteria.

EXPERIMENTAL.

The bacteria used in these experiments were grown from a strain of *B. coli communis* which was obtained from the Pathological Department, Cambridge.

The method by which the bacterial suspension was prepared is described in a paper by Cook and Stephenson [1928].

In general the preparation of bacteria contained approximately 0.3 g. of nitrogen per 100 cc. of stock suspension. Taking the nitrogen as representing 8.3 % of the dry weight of the bacteria [Nicolle and Alilaire, 1909] this would represent an amount of 3.6 g. dry weight of bacteria per 100 cc. of the original suspension. The preparation of resting bacteria retains its activity for several months if kept at 0°. It was however generally used within a fortnight. The toluene-treated organism was prepared according to the method of Quastel and Wooldridge [1928]. It was found that 30 minutes' treatment with toluene at room temperature was sufficient. In preparing the treated organism it was found best to dilute the suspension to the requisite degree before adding the toluene. The suspension was pipetted free of the toluene layer. The results obtained after toluene treatment on different days were always completely reproducible. The substrates used were the purest obtainable. If acids, they were neutralised and brought to the requisite p_H with phosphate buffer solutions.

In the case of dehydrogenations produced by bacteria in the presence of methylene blue, the following solutions were placed in the vacuum tubes: 1 cc. substrate of suitable concentration, 1 cc. of 1 in 5000 methylene blue, 1 cc. water and 2 cc. of buffer at the requisite p_H . To the tubes thus set up was now added 1 cc. of the bacterial suspension diluted to such a concentration that an easily noted reduction time could be obtained. This diluted suspension was kept at 0° during the course of the experiment. Immediately on adding the suspension the stopper was replaced and the tube evacuated for a standard time of 1.5 minutes. The tube was then placed in a thermostat. The time taken for 90 % reduction of the methylene blue was measured. Quastel and Whetham [1924] have shown that the reduction of methylene blue is linear to this point. The oxygen uptake was measured in the Barcroft differential manometer as described by Cook and Stephenson [1928]. The p_H was always controlled at the end of the experiment by electrometric measurement.

In Table I the results given for the cases where fumaric and aspartic acids, nitrates and chlorates are used as hydrogen acceptors are derived from the results of Quastel, Stephenson and Whetham [1925].

Table I summarises the results obtained by the dehydrogenation of the substrates studied in the presence of various hydrogen acceptors with *B. coli*. Series B, C and D are similar dehydrogenations carried out in the presence of inhibitors and will be referred to later.

It is clear that when oxygen is used as a hydrogen acceptor with the untreated organism, dehydrogenation is not the sole process involved. Carbon dioxide is given off. In Table II, column 1 gives the oxygen uptakes and CO₂ outputs for the oxidation of the substrates used with the normal organism (*B. coli*). Column 3 shows the amounts of oxygen and CO₂ corresponding to complete oxidation. The data for oxygen in column 1 are derived in part from the results of Cook and Stephenson [1928].

Table I.

The sign $\times$ indicates that oxidation takes place and is used to indicate the intensity of the reaction. The sign 0 indicates no oxidation, the dashes representing experiments not as yet carried out.

	Series A Normal organism						Series B Organism + 0.001 M KCN			Series C Toluene-treated organism			Series D Organism + sodium fluoride		
	Methylene blue	Fumaric acid	Aspartic acid	Nitrates	Chlorates	Oxygen	Methylene blue	Oxygen	Fumaric acid	Methylene blue	Oxygen	Fumaric acid	Methylene blue	Oxygen	
Glucose	$\times \times \times$	—	—	—	—	$\times \times \times$	$\times \times \times$	?	—	0	0	—	0	0	
Formic acid	$\times \times \times$	—	—	$\times \times \times$	—	$\times \times \times$	$\times \times \times$	*	—	$\times \times \times$	$\times \times$	—	$\times \times \times$	$\times \times \times$	
Acetic acid	$\times \times$	0	0	—	—	$\times \times \times$	$\times \times \times$	0	—	$\times \times$	0	—	$\times \times$	$\times \times \times$	
Lactic acid	$\times \times \times$	$\times$	?	$\times \times \times$	$\times \times$	$\times \times \times$	$\times \times$	0	—	$\times \times \times$	$\times \times$	—	$\times \times$	$\times \times \times$	
Succinic acid	$\times \times \times$	0	0	$\times \times \times$	—	$\times \times \times$	$\times \times$	0	—	$\times \times \times$	$\times \times$	—	$\times \times$	0	
Fumaric acid	0	0	0	$\times \times$	—	$\times \times \times$	0	—	—	0	0	—	0	—	
Pyruvic acid	0	—	—	$\times \times$	—	$\times \times \times$	—	0	—	0	0	—	—	$\times \times$	
Ethyl alcohol	$\times$	—	—	—	—	—	—	0	—	—	—	—	—	—	
Oxalic acid	0	—	—	—	—	0	—	—	—	—	—	—	—	—	
Glycollic acid	$\times$	—	—	—	—	$\times$	—	—	—	—	—	—	—	—	
Malonic acid	0	—	—	—	—	$\times$	—	—	—	—	—	—	—	—	
Alanine	$\times$	0	0	$\times$	—	$\times$	—	—	—	—	—	—	—	—	
Aspartic acid	0	0	0	$\times$	—	$\times$	—	—	—	—	—	—	—	—	
Acetaldehyde	$\times$	—	—	—	—	0	—	—	—	—	—	—	—	—	

* In the case of the oxidation of formic acid by methylene blue in the presence of KCN Quastel and Wooldridge [1927, 2] used much higher concentrations of this substance to bring about the inactivation of the dehydrogenating mechanism than are necessary to stop the mechanism in the presence of oxygen. Concentrations of 0.001 M KCN which effectively stop the oxidation of formic acid by oxygen produce an increase of about 50 % in the reduction time of the methylene blue. It should be noted however that the concentrations of KCN which arrest the oxidation of formic acid by methylene blue have not such a marked effect on the oxidation of glucose, or of succinic and lactic acids. The possible transformation of glucose in the presence of KCN is of great interest. There is no evidence of oxygen uptake, but it is possible that the glucose breaks down as it would anaerobically, despite the presence of oxygen. The products so formed could then undergo oxidation, despite the presence of KCN. This possibility is at present under investigation.

The determination of carbon dioxide. The procedure used for the determination of the amount of CO_2 formed in these experiments is in general principle the same as that used for the determination of the amount of oxygen used in the oxidation of a given substrate [see Cook and Stephenson, 1928]. The experiments were carried out in the Barcroft differential manometer using flat-bottomed cups. The cups were set up as follows.

Left-hand cup 1. 1 cc. buffer (p_{H} 6.0); 1 cc. bacterial suspension.
1 cc. substrate solution of suitable concentration.
No alkali was placed in the inner cup.

Right-hand cup 2. This was set up in the same manner but alkali was placed in the small inner cup.

40 % KOH may be used but it has been found that better removal of the CO_2 takes place if a small roll of filter paper saturated with 5 % KOH is placed in the inner cup [Dixon and Elliott, 1930]. The experiments were carried out at 40° , at which temperature and at the p_{H} used the correction for the solubility of the CO_2 is negligible.

It will be seen that in such an experiment any oxygen uptake due to the substrate itself will be balanced in each cup. In cup 2 the total CO_2 will be removed by the KOH while the movement in the manometer will represent the CO_2 output due to the bacteria and the substrate; a control experiment is

therefore necessary to determine the CO_2 output due to the bacteria alone. This is made in a similar manner to the first experiment.

Cup 1. 1 cc. buffer (p_{H} 6.0); 1 cc. bacterial suspension; 1 cc. water.

No KOH is placed in the inner cup.

Cup 2. The same but KOH is placed in the inner cup.

The movement of the manometer represents the CO_2 output due to the bacteria.

From the total output obtained in the first experiment the amount of CO_2 corresponding to the bacteria used (from the second experiment) is subtracted. The result gives the CO_2 output corresponding to the change of the substrate alone.

The amounts of CO_2 given out as indicated in Table II show that for formate alone does the process correspond to complete oxidation. For the oxidation of lactate and pyruvate, while the amount of oxygen taken up by the pyruvate corresponds to one atom of oxygen less than that used in the oxidation of lactate, the amounts of CO_2 given out are approximately the same. This perhaps indicates that all the lactate passes through pyruvate, this latter then being oxidised.

Table II.

The figures given here represent centimols of gas respired. These values have been calculated from the vol. of dry gas at N.T.P. respired for 1 cc. of $M/100$ solution of substrate and are the mean of several experiments. The sign 0 represents no appreciable oxidation, while the dashes represent experiments which are variable or uncertain and await confirmation.

1 cc. of $M/100$	1 Normal organism		2 Toluene-treated organism		3 Theoretical amounts of gases corresponding to complete oxidation	
	O_2	CO_2	O_2	CO_2	O_2	CO_2
Glucose	3.86	—	0	0	6	6
Lactic acid	1.96	1.87	0.38	0	3	3
Pyruvic acid	1.52	1.85	0	0	2.5	3
Formic acid	0.50	0.97	0.49	0.98	0.5	1
Acetic acid	1.50	1.45	0	0	2	2
Succinic acid	2.40	—	0.41	0	3.5	4
Fumaric acid	1.90	—	0	0	3	4

Toluene-treated organism. Quastel and Wooldridge [1927, 1] observed the interesting fact that, if the resting organism be treated with toluene for a short time and be then removed from the toluene layer, certain dehydrogenations are completely and irreversibly eliminated. The mechanisms effecting the dehydrogenations of formic, lactic and succinic acids are left completely intact. The oxygen-activating mechanism of the cell is also left intact by toluene treatment so that atmospheric oxygen may be used as a hydrogen acceptor.

It will be seen from Table II that with the toluene-treated organism we appear to be considering only those oxidations which are due to dehydrogenases. Thus the organism has no oxygen uptake in the presence of pyruvate or fumarate. The oxygen uptake with formate corresponds to complete oxidation, while that with lactate and succinate, though not as close to the theoretical value for oxidation to pyruvate and fumarate respectively, approximates

to it. It will be seen also that only for formate is there a CO_2 output, this corresponding approximately to the theoretical value. The toluene-treated organism has itself no appreciable oxygen uptake nor does it reduce methylene blue in the absence of substrate. This treated organism forms a very convenient material for the study of these three very important dehydrogenases. The behaviour of the enzymes may be studied *in situ*. All possibility of growth can be ruled out entirely. For other effects which can only be observed when using the toluene-treated organism see Quastel and Wooldridge [1928].

Fig. 1.

Fig. 2.

Fig. 1. The oxidation of $M/300$ lactate by atmospheric oxygen at p_{H} 6.3 and 40° with the normal untreated organism in the presence and absence of oxalate. Oxalate concentration $M/50$.

Fig. 2. The oxidation of $M/150$ formate and $M/150$ lactate by atmospheric oxygen at p_{H} 6.3 and 40° with the toluene-treated organism in the presence and absence of oxalate. Oxalate concentration $M/75$.

Fig. 3. The oxidation of $M/150$ lactate and $M/150$ succinate by atmospheric oxygen at p_{H} 6.3 and 40° with the toluene-treated organism in the presence and absence of malonate. Malonate concentration $M/75$.

Fig. 1 represents the results obtained in the oxidation of lactate by the normal organism in the presence and absence of oxalate. Fig. 2 shows the oxidation of formate and lactate by the toluene-treated organism in presence and absence of oxalate; Fig. 3 represents a similar experiment on the oxidation of lactate and succinate in presence and absence of malonate.

Effect of temperature on the dehydrogenations in the presence of oxygen and methylene blue.

Having thus a material in which the dehydrogenase activities were alone evident, it was suggested by Professor J. B. S. Haldane that a comparison of the velocities of the dehydrogenations produced in the presence of methylene blue and oxygen would yield some results of interest, and that a study of the effect of temperature on the dehydrogenations produced would constitute a good method of attack on the relationship between these two processes. Preliminary experiments were carried out to determine the most suitable p_H at which to carry out this work. It was found that the optimum oxidation of formate was obtained at p_H 6.3, of lactate at p_H 7.4, and of succinate at p_H 7.6. A more complete study of the p_H curves of these dehydrogenations in the presence of oxygen and methylene blue is at present under hand. The experiments with methylene blue were carried out with the same buffer solutions, and as often as possible the experiments were carried out on the same specimens of toluene-treated organism in the same thermostat. Owing to technical difficulties at temperatures above 45° the velocities for oxygen uptake could not be determined with any degree of accuracy, but it appears that there is no further increase in velocity. In the case of the experiments with oxygen it was important that the initial velocities of oxidation should be measured. For this purpose the method used by Keilin [1929] was adopted. The left-hand Barcroft cup was set up as before, the right-hand cup had added buffer, water, and bacterial suspension. The substrate was placed in a small tube which could be hooked on to the side of the potash tube by means of a platinum wire. The apparatus was placed in the thermostat and shaken for 10 minutes, the taps were then closed and the reading taken. The tube containing the reagent was then dropped off and the readings taken every 15 minutes. Table III gives the oxygen uptakes for the consecutive 15 minute readings for formate, lactate and succinate at various temperatures.

Table III.

Temperature	Donator	O ₂ uptake (mm. ³) at the end of			
		15 mins.	30 mins.	45 mins.	60 mins.
14°	Formate <i>M</i> /150	12.7	58	101.7	141.8
14.4°	Lactate <i>M</i> /60	5.4	11.75	16.55	21.55
15.6°	Succinate <i>M</i> /60	12.6	23	33.8	44.2
23°	Formate	55.8	145.5	192.4	210.1
25°	Lactate	11.3	21.2	29.8	39.05
	Succinate	21.8	49.9	75.3	97.2
28.5°	Formate	79	169.1	223.2	—
30°	Lactate	17.5	38.2	56.6	75.2
	Succinate	40.4	71.7	95.2	115.5
38°	Formate	90	173	192	198
40°	Lactate	25.1	47	—	—
	Succinate	51.1	85.4	112.4	136.2
45°	Formate	44.5	170.5	206.3	213.3
	Lactate	27.9	45.7	62	77
	Succinate	61	102.1	137.5	169

The velocities were calculated in terms of millimols of substrate oxidised per hour. It is convenient to have a simple formula so that the velocity of oxidation of any donator for a standard enzyme concentration may be calculated.

Oxygen uptake. Taking B as the oxygen uptake in mm.^3 , in T minutes in the presence of a bacterial suspension that has been made up to V cc., the rate of oxidation for the undiluted enzyme preparation may be expressed as

$$\frac{B \times 60 \times V}{11.2 \times 1000 \times T} \text{ millimols per hour.}$$

Methylene blue reduction.

The mol. wt. of methylene blue (see below) has been taken as 325. The concentration adopted throughout these experiments was 1 in 5000. The reduction was usually taken to 90 %. The velocity of oxidation of the substrate may be expressed thus, where D represents the degree of reduction of the methylene blue:

$$\frac{D \times 60 \times 0.2 \times 1000 \times V}{325 \times T \times 1000} \text{ or } \frac{DV}{27.08 \times T}.$$

The figure taken for the mol. wt. of methylene blue may be criticised on the ground that samples of methylene blue vary quite considerably in their purity. The sample used here was the purest obtainable from British Drug Houses. Clark [1928] has dealt with the subject of the purity of methylene blue. It will be seen however, that a calculation of the velocities on the assumption that the apparent gram-molecular weight was 400 or that the sample contained a fair percentage of impurity would not appreciably affect the position of the reduction curve on the logarithmic scale. In fact a closer correspondence between the velocity curves for oxygen and methylene blue at the lower temperatures would be obtained.

Table IV gives the oxygen uptakes for the initial 30 minutes and reduction times of methylene blue in the presence of a constant enzyme concentration for formic dehydrogenation at p_{H} 6.3.

Table IV.

Oxidation of $M/150$ formate at p_{H} 6.3.

Temperature	O_2 mm. ³ (dry at N.T.P.) for initial 30 mins.	Time for complete reduction of 1 cc. of 1/5000 M.B. (mins.)
0°	—	53.5
7	21.3	26
13.5	—	14.75
14	58	—
23	145.5	—
26.5	—	6.88
28.5	169.1	—
32.5	—	4.88
38	173	—
45	170.5	2.75
50	—	2.50
55	—	2.25

The bacterial suspension in the case of oxygen experiments represents a dilution of 1 in 12 and for the methylene blue of 1 in 40.

The reduction of methylene blue was taken in this case to completion. With the oxidation of formate by methylene blue the reduction times are so short that complete reduction is easily noted even at low temperatures.

The results given in the tables represent the mean of four experiments. Experiments conducted with another strain of *B. coli* obtained from the National Collection of Type Cultures gave similar results.

Table V gives the results for the oxidation of *M/60* lactate and Table VI for the oxidation of *M/60* succinate.

Figs. 4, 5 and 6 represent the results obtained by plotting the velocities of the oxidations against the reciprocals of the absolute temperatures of the experiments.

Table V.

Oxidation of *M/60* lactate at p_H 7.4. Bacterial suspension diluted in oxygen experiments 1/12 and for methylene blue experiments 1/20.

Temperature	O ₂ mm. ³ per 30 mins.	Time for 90 % reduction of M.B. (mins.)
14.4°	11.15	—
15.2°	—	39.3
25°	21.2	16.3
30°	38.2	10.75
35°	—	7.75
40°	47	4.8
45°	45.7	4.0

Table VI.

Oxidation of *M/60* succinate at p_H 7.6. Bacterial suspension diluted as for lactate oxidation.

Temperature	O ₂ mm. ³ per 30 mins.	Time for 90 % reduction of M.B. (mins.)
15.6°	23	92.25
25°	49.9	26.25
30°	71.7	16.75
40°	85.4	8.08
45°	102.1	5.50
50°	—	4.25
55°	—	3.75

As will be seen from Figs. 4, 5 and 6 the log. of the velocity of reduction of methylene blue by formate, lactate and succinate is a nearly linear function of the reciprocal of the absolute temperature until enzyme destruction occurs at temperatures above 55°. In the case of oxygen it will be noted that the velocity of its reduction does not increase when a certain temperature is exceeded in the cases of formate and lactate. In other words the rate of oxygen reduction is now the limiting factor. Succinate oxidation by oxygen is accelerated by increase of temperature, but not in a proportional amount to methylene blue reduction. The oxidation of succinate is interesting in that there is such a considerable difference in the velocities at low temperatures between oxygen and methylene blue. A similar result was obtained for muscle succinoxidase by Fleisch [1924]. It appears that in this case there is some considerable

difference between the mechanisms of oxygen and methylene blue activations. This subject is at present under investigation.

Fig. 4.

Fig. 5.

Fig. 4. The oxidation of *M*/150 formate at p_H 6.3 by equivalent amounts of toluene-treated organism in the presence of methylene blue and oxygen at various temperatures.

Ordinate: log. velocity, the velocity being expressed in millimols of formic acid oxidised per hour.

Abscissa: reciprocal of absolute temperature.

Fig. 5. The oxidation of *M*/60 lactate at p_H 7.4.

Fig. 6. The oxidation of *M*/60 succinate at p_H 7.6.
Ordinate and abscissa as in Figs. 4 and 5.

It may be argued that at higher temperatures enzyme destruction was taking place, thus accounting for the limiting velocity. There is however no considerable falling off in velocity. In Table III the oxygen uptakes for the consecutive 15 minute readings at different temperatures are given. In the case of succinate there appears to be some difference in the initial 15 minutes reading for the experiments at 30°, 40°, and 45°. No satisfactory explanation can be offered for this phenomenon. It is probable that the oxygen-activating mechanism in the case of succinate is rather more complex than for the cases of formate and lactate.

To test the possibility of enzyme destruction at 45° a bacterial suspension was exposed to this temperature with continuous aeration for 2 hours. Experiments were then carried out on the oxygen uptake and methylene blue reduc-

tion for formate and lactate dehydrogenations. A very slight decrease in velocity was noted for the two cases.

As shown in Fig. 2 the mechanism causing the dehydrogenation of lactic acid may be competed for by oxalic acid in the presence of oxygen. It was thought of interest to study the effect of increasing concentrations of oxalate on the velocity of oxidation of lactate by methylene blue and oxygen at a temperature at which the activation of oxygen was the limiting factor. Fig. 7 shows the results

Fig. 7. The oxidation of *M/60* lactate at p_H 7.4 and 40° by equivalent amounts of toluene-treated organism in the presence of oxygen and methylene blue with increasing concentrations of oxalate.

Ordinate: velocity. Abscissa: log. molar oxalate concentration.

Upper curve: methylene blue reduction. Lower curve: oxygen reduction.

obtained. It will be seen that for the case of methylene blue an adsorption curve of the Michaelis type is obtained. For oxygen there is at first no effect, oxygen being the limiting factor; after the concentration of oxalate has reached a certain value the oxalate competes for the lactic dehydrogenase enzyme. This limiting factor for the oxygen is of great interest and it was thought that diffusion of oxygen might be the cause. Experiments conducted in pure oxygen show, however, no appreciable increase in velocity.

Table VII.

Temperature	Donator	O ₂ uptake (mm. ³) in pure O ₂ at the end of			
		15 mins.	30 mins.	45 mins.	60 mins.
16°	<i>M/60</i> lactate	9	13.1	15.9	20
	<i>M/60</i> succinate	11.35	21.65	31.25	39.05
36°	Lactate	25.7	48.4	61.9	—
	Succinate	31.9	56.5	77.3	—

Table VII gives the results obtained in pure oxygen at two temperatures with the oxidation of *M/60* lactate and *M/60* succinate.

These figures may be compared with those in Table III in which the atmospheric oxygen uptake was measured. The concentration of bacterial suspension was the same in the two instances. Experiments on the oxidation of the three substrates in low tensions of oxygen (4–5 %) at low and high temperatures gave identical results with the air controls.

As has been pointed out the toluene-treated organism was particularly studied, as those oxidations which are due to the dehydrogenases were alone evident. If the untreated organism was used in the oxidation of lactate and succinate, after dehydrogenation the pyruvate and fumarate formed would be broken down further, these reactions being accelerated by increase in temperature so that no data of any significance could be obtained. Table II shows that with the untreated organism and formate there is evidence of complete dehydrogenation. Experiments were carried out on the oxidation of formate by the untreated organism in the presence of oxygen. The results are expressed graphically in Fig. 8. It will be seen that the velocity of oxidation of formate is

Fig. 8.

Fig. 8. The oxidation of *M/150* formate at p_H 6.0 by equivalent amounts of the normal and toluene-treated organism in the presence of atmospheric oxygen at various temperatures. Ordinate and abscissa as for Fig. 4.

Fig. 9.

Fig. 9. The oxidation of *M/120* lactate and formate at p_H 7.4 and 40°, and the oxidation of the combined substrates. The curve shown in dotted line represents the theoretical curve obtained on summing the values for the first two curves.

Ordinate and abscissa as for Figs. 1 and 2.

slightly increased with toluene treatment of the organism. The difference however is not great and may possibly be concerned with differences in cell permeability after toluene treatment. It will also be seen that the effect of increase of temperature in the two cases is the same. There appears to be also a limiting factor for oxygen reduction with the normal organism. This experiment clearly indicates that toluene treatment of the organism does not damage the oxidase mechanism.

It was thought of interest to see if the dehydrogenases present were specific

in their action. With this object experiments were conducted on the oxidation of formate and lactate alone at p_H 7.4 and 40° and on a mixture of these two substrates in quantities the sums of those used in the former experiments. Fig. 9 shows the results obtained. It will be seen that the curve obtained is approximately identical with the theoretical curve obtained by adding the values obtained for the separate substrates. The slightly lower value obtained for the experimental curve is interesting in that an equilibrium may be attained between the pyruvic acid formed from the lactic acid and the hydrogen formed from the formic acid.

At the present stage it is not possible to attempt an interpretation of the above results. It is evident that the oxidase system must be of considerable complexity. The reactions of the above system to carbon monoxide and HCN which are at present under investigation with Professor J. B. S. Haldane and Mr L. W. Mapson indicate a complexity of respiratory enzymes. It is interesting to note that Hartridge and Roughton [1925] found that the rate of combination of haemoglobin with oxygen has no temperature coefficient. The Blackman effect in photosynthesis is also of a similar nature to the system studied here.

SUMMARY.

A comparison has been made of the oxidation of various substrates in the presence of oxygen and methylene blue and other hydrogen acceptors by suspensions of normal and toluene-treated *B. coli*.

The toluene-treated organism is of importance as the oxidations which are due to the formic, lactic and succinic dehydrogenases may be studied apart from other secondary oxidative processes which are eliminated by toluene treatment. The oxygen-activating mechanism appears to be unaffected by toluene treatment.

A study of the effect of temperature on the dehydrogenations produced by the toluene-treated organism showed that for formate and lactate the velocities of oxidation by methylene blue and oxygen for the lower temperatures were nearly equal. Above a certain temperature the velocity of oxygen reduction did not increase.

In the dehydrogenation of succinate there is considerable difference between the velocities of oxidation by oxygen and methylene blue. In this case a limiting temperature for the oxygen reduction is not so readily attained.

The "competitive" action of oxalate and malonate for the lactic and succinic dehydrogenase mechanisms respectively is demonstrated for the oxygen acceptor system.

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REFERENCES.

- Clark (1928). Studies on oxidation-reduction, r-x (Washington).
Cook and Stephenson (1928). *Biochem. J.* **22**, 1368.
Dixon and Elliott (1930). *Biochem. J.* **24**, 820.
Fleisch (1924). *Biochem. J.* **18**, 294.
Hartridge and Roughton (1925). *Proc. Roy. Soc. Lond. A* **107**, 654.
Keilin (1929). *Proc. Roy. Soc. Lond. B* **98**, 312.
Nicolle and Alilaire (1909). *Ann. Inst. Past.* **23**, 547.
Quastel (1928). *J. Hyg.* **28**, 139.
—— and Stephenson (1925). *Biochem. J.* **19**, 660.
—— Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
—— and Whetham (1924). *Biochem. J.* **18**, 519.
—— ——— (1925). *Biochem. J.* **19**, 645.
—— and Wooldridge (1927, 1). *Biochem. J.* **21**, 148.
—— ——— (1927, 2). *Biochem. J.* **21**, 1224.
—— ——— (1928). *Biochem. J.* **22**, 689.