LXX. THE EQUILIBRIA EXISTING BETWEEN SUCCINIC, FUMARIC, AND MALIC ACIDS IN THE PRESENCE OF RESTING BACTERIA.

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It was observed in a series of preliminary experiments described in a previous paper [Quastel, 1924] that the rate of decolorisation of methylene blue by resting *B. coli* and *B. pyocyaneus* was considerably accelerated by the presence of sodium succinate. These observations have been extended with the result that it is clear that a reversible equilibrium

succinic acid + methylene blue $\rightleftharpoons$ fumaric acid + leuco-methylene blue
exists in the presence of these resting organisms.

By resting organism we mean an organism which has been grown for two days in tryptic broth [Cole and Onslow, 1916], separated by centrifuging, thoroughly washed with normal saline, made up to a thick emulsion with saline, and finally very well aerated, which serves to produce a fairly homogeneous emulsion and to remove any traces of easily oxidisable material. Such an emulsion has been used for the investigations to be described and is always employed under such conditions that growth does not occur. The reactions brought about by the organism under these conditions we consider to be reactions produced by the resting organism. We have hesitated in committing ourselves to the term "resting organism" in view of the designation of the sporing stage of certain organisms as a resting stage. We feel, however, that no confusion need necessarily arise. The term resting organism as we have defined it is useful for comparison with resting muscle and other tissues.

Our investigations have been confined so far almost exclusively to resting *B. coli comm.*, but qualitative experiments show that resting *B. pyocyaneus* has similar properties.

Measurements of the velocity of reduction of methylene blue were made under anaerobic conditions in vacuum tubes such as those used by Thunberg [1920] and later by Hopkins and Dixon [1922]. The succinic, fumaric and malic acids were always neutralised with caustic soda to a p_H of 7.2. Care was taken that the solutions under investigation were properly buffered. The observations which are recorded below are typical of the average of those made. They are only a small proportion of the total number.

Our first inquiries turned on the properties of the enzyme, existing in the resting organism, which is capable of transferring hydrogen from succinic

acid to methylene blue. We found that the activity of the organism varied considerably with its history, generally becoming more marked after standing for one or two days. If allowed to stand too long, the organism itself acquires increased reducing properties which make it undesirable for use. We have employed two strains of *B. coli* and several growths from each strain. These will be referred to as coli 1, coli 2, etc. In all cases, the reduction velocities can only be regarded from a comparative point of view, since the various emulsions differed in the total quantity of organism present, but for any one series of experiments the same emulsion was always used.

As instances of the reducing activity of the organism with and without succinic acid, the following observations are typical.

Tubes...	1	2	3	4
cc. of organism (coli 1) ...	1	1	1	1
„ 7.6 buffer ...	1	1	1	1
„ 1 % sodium succinate...	1	0	1	0
„ water ...	0	1	0	1
„ methylene blue (1/5000)	1	1	1	1
Temperature ...	38°	38°	60°	60°
Time for decolorisation ...	35 mins.	Not decolorised in 16 hrs.	6 mins.	1 hour

Potassium cyanide and sodium fluoride do not inhibit the reduction of methylene blue by the organism in presence of succinate.

Effect of hydrogen ion concentration.

It was at once evident from observations on the effect of varying hydrogen ion concentration on the times of reduction that two effects were superimposed upon one another, (1) an effect upon the physical structure of the organism, (2) an effect on the chemical reaction. Below a p_H of 5.0 no apparent reduction could be obtained, and the organism appeared to be coagulated. Above p_H 6.0 there was rapid increase of the velocity of reduction with decrease of hydrogen ion concentration until a p_H of 9.0, when a further increase in p_H did not bring about any rise in reaction velocity. We have not investigated the effect of a p_H above 10.0, but we have reason to believe that in alkaline solutions the organism suffers some change, which affects its reducing activity considerably. The most suitable p_H for a high reduction velocity without fear of inactivation of the organism is about 7.4.

The results of a series of experiments on the effect of hydrogen ion concentration on reduction times are expressed graphically in Fig. 1. The solutions used consisted of 1 cc. of organism (coli 2), 1 cc. of 1 % sodium succinate, 1 cc. of methylene blue (1/5000), and 1 cc. of buffer of varying p_H . The temperature throughout was 45°.

It is interesting to compare these results with those of Ohlsson [1921] who finds with succinodehydrogenase extracted from horse muscle an optimum p_H of about 9.0 above which there is a sharp decrease in activity. The enzyme used by Ohlsson was thus more sensitive to low hydrogen ion concentrations than the resting organism.

When phosphate buffer was replaced by borate buffer of the same p_H , there was no difference in the velocity of reduction.

That each emulsion of organism was homogeneous and that equal volumes could be regarded as possessing the same quantity of enzyme was demonstrated by the fact that reduction times of equal volumes of emulsion for the same quantities of methylene blue and succinate were identical.

Fig. 1.

Effect of temperature at constant hydrogen ion concentration.

Here, as in the previous experiments, two superimposed effects (1) on the physical condition of the enzyme, (2) on the chemical reaction, are obvious. The reduction velocity increases with increase of temperature, the temperature coefficient between 30° and 60° being 2.1 (see Fig. 2). At 70° there is an extremely rapid reduction for two or three minutes, which suddenly ceases before complete decolorisation, and this must be associated with the fact that the organism has become coagulated. It appears, therefore, that at this temperature, the increased velocity of destruction of the enzyme (that is, of retardation of reduction) becomes of the same order as the increased velocity of the reaction between the succinate and the methylene blue. The following observations on the effect of exposing the resting organism to a high temperature will make this clear.

	Tubes...	1	2	3	4
cc. of organism (coli 3) ...	...	1	1	1	1
„ 7.6 buffer ...	...	1	1	1	1
„ 1 % sodium succinate ...	...	1	1	1	1
„ methylene blue (1/5000) ...	...	1	1	1	1
Temperature at which organism was previously heated for 10 mins. ...	...	60°	—	70°	—
Temperature of thermostat ...	...	60°	60°	60°	60°
Time for decolorisation with heated organism	...	9 mins.	—	No decolorisation	—
Time for decolorisation with unheated organism (control) ...	...	—	4½ mins.	—	4 mins. 40 secs.

We find that a temperature of 45° is very suitable for a high velocity of reduction without risk of injury to the organism, and all subsequent work was consequently carried out at this temperature.

Fig. 2.

Effect of concentration of organism.

The rate of reduction is roughly directly proportional to the concentration of organism present.

Tubes...	1	2	3	4
cc. of organism (coli 4) ...	3	2	1	0.5
„ 7.3 buffer ...	1	1	1	1
„ 1 % sodium succinate...	1	1	1	1
„ methylene blue (1/5000)	1	1	1	1
„ saline	0	1	2	2.5
Time for decolorisation ...	2½ mins.	3½ mins.	7 mins.	13 mins.

All attempts to extract a succinodehydrogenase from the organism by the means which have been successful in the case of muscle have so far failed. Shaking the organism with $N/200$ NaOH or with $M/15$ $(\text{NH}_4)_2\text{HPO}_4$ is not effective in removing the reducing factor.

Fig. 3.

The rate of decolorisation of methylene blue.

Fig. 3 is a typical example of the results obtained in a series of experiments on the rate of reduction of methylene blue by sodium succinate. A mixture of 1 cc. of organism, 1 cc. of 7.2 buffer, 1 cc. of 1 % sodium succinate, and 1 cc. of methylene blue (1/5000) was placed in a vacuum tube and allowed to reduce under anaerobic conditions at 45° . The methylene blue was estimated at intervals by colorimetric comparison with standard solutions of methylene blue, to which organism had been added in order to make the opacities of the vacuum tube and the comparison tube as nearly equal as possible.

The rate of decolorisation of a solution *in vacuo* was not different from that of one in an atmosphere of nitrogen.

Reversibility.

It was shown in an earlier communication [Quastel, 1924] that fumaric acid (as the sodium salt) provides a very efficient nutrient supply to *B. pyocyaneus* and *B. coli* and it was shown too that, in the case of the former

organism, pyruvic acid was the first demonstrable product in the fermentation, oxalacetic acid being the most likely precursor of pyruvic acid. Evidence was brought forward to demonstrate that the unsaturated linkage was capable of fixing oxygen to produce a ketonic structure, the process being extremely rapid with the growing organism. Now it seemed possible that this process might be made to occur anaerobically by using a suitable hydrogen acceptor instead of atmospheric oxygen; that fumaric acid and, say, methylene blue, might act as oxygen acceptor and hydrogen acceptor respectively with water as the donator of both the oxygen and the hydrogen. This was tested, and it was found that fumaric acid in presence of the resting organism produced no reduction of methylene blue whatever. What was actually found was a definite inhibition of the normal reducing capacity of the organism alone. Investigating this inhibition further, it was found that fumaric acid inhibits the reduction of methylene blue by sodium succinate in presence of resting organism, in much the same way as has been demonstrated by Wishart [1923] for the case of muscle. No claims have been made by the workers on resting muscle that an equilibrium exists between succinic acid, fumaric acid, methylene blue, and its leuco-derivative, but our observations on the relative degrees of inhibition exerted by varying concentrations of fumarate led us to the belief that a true, reversible equilibrium does exist. This opinion was strengthened, moreover, by finding that maleic acid has no similar effect to fumaric acid, which at once gives the fumaric inhibition a specific character. Concentrations of maleic acid (neutralised by sodium hydroxide) varying from 0.1 % to 10 % had no apparent effect on the decolorisation of methylene blue by succinate in presence of resting organism.

The fumaric acid inhibition was then investigated in the following ways: determination of the effect of sodium fumarate on *leuco*-methylene blue, (1) with resting organism, (2) with dead organism, (3) without organism.

(1) In order to show that the inhibition was actually due to a reaction between fumarate and *leuco*-methylene blue, the following experiment was devised. Use was made of the fact that the resting organism is capable of reducing oxidised glutathione to the —SH compound, the reduced glutathione itself reducing methylene blue. Thus in presence of a trace of oxidised glutathione the resting organism very readily reduces methylene blue. If now to the system—resting organism, oxidised glutathione, and methylene blue—sodium fumarate be added (in excess of the glutathione present), the methylene blue is no longer completely reduced. The following experiment demonstrates this.

	Tubes...	1	2	3
cc. of organism (coli 5) ...	...	2	2	2
„ 7.2 buffer ...	...	1	1	1
„ methylene blue (1/5000) ...	...	0.5	0.5	0.5
„ 1 % neutral oxidised glutathione	...	—	0.5	0.5
„ 1 % sodium fumarate ...	...	—	—	1.0
„ of saline ...	...	1.5	1.0	—
Time for decolorisation	...	Not decolorised	11 mins.	Not completely decolorised in 1 hr.

Similarly, sodium fumarate has an inhibitive action on the reduction of methylene blue by cysteine in presence of resting organism.

	Tubes...	1	2
cc. of organism (coli 5) ...	...	1	1
„ 7.2 buffer ...	...	1	1
„ methylene blue (1/5000) ...	...	1	1
„ neutral cysteine solution (0.3 %) ...	...	0.5	0.5
„ 1 % sodium fumarate ...	...	—	1
„ saline ...	...	1	—
Time for decolorisation ...	...	15 mins.	Not completely decolorised in 1 hr.

The most conclusive manner of demonstrating the oxidising action of sodium fumarate on *leuco*-methylene blue in presence of resting organism is to allow a solution of sodium fumarate to react *in vacuo* with a mixture containing the *leuco*-dyestuff and the resting organism. The white solution gradually becomes bluer until it reaches a definite intensity of colour depending on the concentration of fumaric acid, and this colour remains practically permanent. The experiment is best done by making use of an inverted U tube which can be evacuated. The two limbs of the U tube employed were 7" by $\frac{3}{4}$ ", the neck being 3" long. The neck was plugged by a short piece of rubber tubing containing a length of glass tube to which was affixed an air-tight tap. The tap was always well greased and a vacuum could be maintained almost indefinitely in the tube. In one limb of the tube a mixture of resting organism, buffer solution, sodium succinate solution, and methylene blue is placed, in the other limb a solution of sodium fumarate. If care be taken in the quantities of material used, in the evacuation, and in the subsequent proceedings, there need be no bubbling of the solution in one limb into that in the other. The risk of the solutions mixing by ebullition may be, however, completely eliminated by filling the evacuated tube with nitrogen. The evacuated (or nitrogen filled) tube is left in the thermostat until reduction of the methylene blue by the succinate has been accomplished. The two solutions are then mixed and in a minute or two the gradual blueing of the solution becomes apparent. Sodium maleate has no such effect. If the evacuation has not been sufficiently complete, there may be a slight immediate blueing, due to a trace of oxygen left in the solution. On standing for about five minutes, however, this colour disappears as the succinate exerts its reducing power. A solution which has become blue owing to addition of sodium fumarate remains blue for an almost indefinite period.

(2) It was then decided to ascertain whether the reaction between the *leuco*-methylene blue and the fumarate depended on a thermolabile enzyme. Use was again made of the vacuum U tube. In one limb the succinate + resting organism + methylene blue mixture was placed as before, and in the other the solution of fumarate. The tube was evacuated, filled with nitrogen, and allowed to stand in the thermostat until reduction of the methylene blue was accomplished. It was then placed in a water-bath and the temperature of the bath was gradually brought up to 100°. The pressure

of gas was relieved by opening the tube to atmospheric pressure at intervals. After exposure to this temperature for a quarter of an hour, the tube was allowed to cool, the tap being closed so that no oxygen entered as cooling proceeded. The tube was then re-evacuated to remove any traces of oxygen that might have entered, the solutions in the two limbs were mixed, and the tube was placed in the thermostat. No blueing whatever occurred.

(3) Definitely to show that there was no action between sodium fumarate and *leuco*-methylene blue, the following experiment was performed. In one limb of the vacuum U tube was placed a mixture of cysteine solution, buffer solution, and methylene blue, and in the other sodium fumarate solution in a concentration considerably greater than that of the cysteine. The tube was evacuated and placed in the thermostat, and when the methylene blue had been reduced by the cysteine, the solutions were mixed. There was no oxidation of the *leuco*-methylene blue. When this experiment was repeated in the presence of resting organism, a slight but definite blueing of the *leuco*-methylene blue did occur.

It must be concluded from these experiments that just as sodium succinate reduces methylene blue in the presence of resting organism, so does sodium fumarate oxidise *leuco*-methylene blue in presence of the organism.

The matter was investigated from a quantitative aspect. It was desirable to show first that the equilibrium attained by a mixture of known quantities of succinate, fumarate, and methylene blue, was the same as that reached after the addition of the same quantity of fumarate to a solution of the same quantities of methylene blue and succinate, complete reduction of the dyestuff having been allowed to occur. In a vacuum tube was placed the following mixture: 1 cc. coli 6, 1 cc. 7.2 buffer, 1 cc. methylene blue (1/5000), 0.25 cc. 1 % sodium succinate, 1 cc. 1 % sodium fumarate, and the rate of decolorisation was determined by colorimetric comparison. Again, in one limb of a vacuum U tube was placed the mixture of 1 cc. coli 6, 1 cc. 7.2 buffer, 1 cc. methylene blue (1/5000), 0.25 cc. 1 % sodium succinate, and in the other limb 1 cc. 1 % sodium fumarate. Reduction of the methylene blue was allowed to proceed to completion, the solutions in the two limbs were mixed, and the rate of blueing observed by colorimetric comparison. The results are expressed graphically in Fig. 4. It will be seen that the same equilibrium point is approached asymptotically by both solutions.

Assuming that the equilibrium

exists in presence of the resting organism, it follows that at equilibrium conditions will be represented by

$$\frac{[\text{fumaric acid}][\text{leuco-methylene blue}]}{[\text{succinic acid}][\text{methylene blue}]} = K \text{ (equilibrium constant),}$$

where the square brackets denote molecular concentrations at equilibrium. If we begin with a molecules of sodium succinate, b of methylene blue, and

x of sodium fumarate, and if at equilibrium there are n molecules of methylene blue present, we have

$$\frac{an - n(b-n)}{x(b-n) + (b-n)^2} = \frac{1}{K}.$$

This equilibrium constant will be independent of the amount of resting organism so long as conditions of true catalysis hold. If, however, some material of the resting organism enters into one side of the equation and not into the other, or if different quantities of the same material enter into the two sides of the equation, K will vary with the quantity of organism present.

Fig. 4.

A large number of observations were made on the amounts of methylene blue left at equilibrium with varying initial quantities of succinate, fumarate, organism, and methylene blue. The hydrogen ion concentration and temperature were kept constant throughout. The solutions were mixed in a vacuum tube, evacuated as quickly as possible, and left in the thermostat for one hour, when it was found that equilibrium had been reached. Generally, with an active preparation of *B. coli*, ten to twenty minutes were really sufficient. The rates at which equilibrium was reached varied with the quantities of organism present. The methylene blue was estimated by colorimetric comparison, the method being to add a known quantity of methylene blue to a definite quantity of the resting organism in a tube precisely similar to the vacuum tube. Water was then added to the comparison tube from a burette until the intensity of colour of methylene blue in the vacuum and comparison tubes was the same. Knowing the total volumes in both tubes, it is easy to calculate the amount of methylene blue in the vacuum tube. Control experiments showed that with practice at matching the intensities of colour the maximum error does not exceed 5 %. The greatest errors occur with very high and very low concentrations of methylene blue. The difficulty

in matching the colours lies chiefly with the opacity due to the organism and care must be taken to make the opacities roughly the same by addition of suitable known quantities of organism to the comparison tube. It is difficult to obtain accurate readings with *B. pyocyaneus* owing to the pigment in this organism. *B. coli* if it has been properly washed has only the very slightest yellowish tinge which does not affect readings appreciably. It may be mentioned here that *B. coli* is far easier to handle than *B. pyocyaneus* from the point of view of physical characteristics and this constituted one of the reasons for our dealing almost exclusively with *B. coli*.

Table I gives examples of the results obtained with varying concentrations of succinate, fumarate, methylene blue, and organism. The percentage reduction found and that theoretically deduced for a definite value of K is given in each case. We have taken $K = 3.0$ and it will be seen that the agreement between the observed and the calculated reductions is sufficiently good to prove that the equilibrium postulated above does actually exist.

Table I. *Temperature throughout = 45°. $p_H = 7.2$, $K = 3.0$.*

Amount of organism	cc. of 1 % sodium succinate	cc. of 1 % sodium fumarate	cc. of methylene blue (1/5000)	cc. of methylene blue remaining at equilibrium	% reduction found	% reduction calculated
2 cc. coli 6	0.1	1.0	0.75	0.58	22.6	22.0
"	0.25	1.0	0.8	0.467	41.7	42.0
"	0.25	1.0	1.0	0.583	41.7	42.0
"	0.5	1.0	0.5	0.207	58.6	59.8
2 cc. coli 7	1.0	1.0	1.0	0.245	75.5	75.0
"	1.0	0.5	1.0	0.166	83.6	85.7
"	1.0	0.75	1.0	0.202	79.8	80.0
"	1.0	1.5	1.0	0.515	68.5	66.6
1 cc. coli 7	1.0	1.0	1.0	0.260	74.0	75.0
"	0.75	1.25	1.0	0.380	62.0	64.4
"	0.5	1.5	1.0	0.50	50.0	50.0
"	1.5	0.5	1.0	0.08	92.0	90.0
"	2.0	1.0	1.0	0.124	87.6	85.7
2 cc. coli 7	0.5	1.5	1.0	0.50	50.0	50.0
"	1.5	0.5	1.0	0.09	91.0	90.0
"	0.25	1.75	1.0	0.67	33.0	30.0
"	1.25	0.75	1.0	0.17	83.0	83.3
1 cc. coli 8	1.0	3.0	1.0	0.486	51.4	50.0
"	1.0	2.0	1.0	0.412	58.8	60.0
"	1.0	0.25	1.0	0.104	89.6	92.3
2 cc. coli 8	*5.0	1.0	2.5	0.066	97.2	93.6
"	*5.0	1.5	2.5	0.176	92.9	92.3
"	*5.0	0.0	15.0	0.183	98.7	99.0
"	*2.5	2.0	2.5	0.494	80.2	79.2
"	*2.5	2.0	5.0	0.444	91.1	89.6

* In these cases more concentrated solutions of sodium succinate and methylene blue were used.

It will be seen on examination of the table that K is independent of the quantity of organism present.

Isolation of fumaric acid.

For isolation of fumaric acid from succinic acid and methylene blue in presence of organism the following method proved to be satisfactory. The

resting organism (*B. coli*) from a 48 hours' growth on three Roux bottles of tryptic broth (each containing 150 cc.) was placed in a vacuum flask with 50 cc. of a phosphate buffer at p_H 7.2, and solutions containing 2 g. of sodium succinate, and 1 g. of methylene blue, the total volume being 350 cc. The flask was evacuated and left at 37° for 24 hours. After this time the flask was opened and Merck's charcoal was added. The solution was filtered, the residue well washed and the filtrate and washings evaporated to low bulk on the water-bath. The liquid was acidified with sulphuric acid (to 10 % acid) and extracted well with ether. The ethereal extract was filtered through dry paper and evaporated. The crude acids which were left as residue were dissolved in a small quantity of water and to the solution was added silver nitrate solution. The white precipitate was well washed, suspended in warm water, and treated with sulphuretted hydrogen. The silver sulphide was filtered off, and the filtrate evaporated to dryness. About 80 mg. of crude acid were obtained from which 60 mg. of a pure specimen of fumaric acid were prepared. This does not represent the total yield of fumaric acid, for the method given above, though fairly satisfactory, is not strictly quantitative. The fumaric acid was identified by its melting point and a mixed melting point with a pure specimen of fumaric acid. It did not melt at 200° in an open melting point tube but showed signs of sublimation. In a closed melting point tube it melted at 280°. Its behaviour was not altered by admixture with an authentic specimen of fumaric acid. A known quantity was titrated with standard alkali from a micro-burette: 0.0143 g. of the acid dissolved in water required 2.00 cc. of 0.1163 *N* alkali for neutralisation, a quantity equivalent to 0.0145 g. of fumaric acid. The acid was precipitated from aqueous solution by silver nitrate solution and decolorised potassium permanganate in the cold.

It was further identified by making use of the equilibrium equation given above. 0.0052 g. of the acid was neutralised with caustic soda and added to a mixture of 2 cc. coli 9, 1 cc. 7.2 buffer, 1 cc. methylene blue (1/5000), 0.2 cc. 1 % sodium succinate, in a vacuum tube. The tube was evacuated and the equilibrium point reached was estimated by colorimetric comparison of the methylene blue. This was equivalent to 0.23 cc. of 1/5000 methylene blue, which, on substituting in the equation above with $K = 3.0$ gives 0.0049 g. of fumaric acid.

Malic acid.

On examining the effect of malic acid (as the sodium salt of *l*-malic acid) on the reduction of methylene blue in presence of the resting organism it was found

- (1) that malic acid resembles fumaric acid in not reducing methylene blue in presence of resting organism;
- (2) that malic acid slightly but definitely inhibits the decolorisation of methylene blue by succinate in the presence of resting organism.

The following experiments demonstrate this.

		Tubes...	1	2	3	4	5
(1)							
cc. of organism (coli 10)	...	1	1	1	1	1	1
„ 7.2 buffer	...	1	1	1	1	1	1
„ methylene blue (1/5000)	...	1	1	1	1	1	1
„ 1 % sodium succinate	...	—	0.5	—	0.5	0.5	0.5
„ 1 % malic acid (as sodium <i>l</i> -malate)	...	—	—	1	0.5	—	—
„ 1 % sodium fumarate	...	—	—	—	—	—	0.5
„ saline	...	1	0.5	—	—	—	—
Time for decolorisation	...	Not de- colorised	12 mins.	Not de- colorised	Nearly decolorised in 12 mins., colour apparently stable	Reaches equili- brium	
(2)							
			Tubes...	1	2		
cc. of organism (coli 10)	...	...	...	1	1		
„ 7.2 buffer	...	...	...	1	1		
„ methylene blue (1/5000)	...	...	...	1	1		
„ 1 % sodium succinate	...	...	...	0.2	0.4		
„ 1 % malic acid (as sodium <i>l</i> -malate)	...	...	...	1	1		
Equilibrium reached (expressed in cc. of 1/5000 methylene blue)	...	...	...	0.17	0.07 (observed after 33 mins.)		

It will be shown in a subsequent communication that *B. pyocyaneus* ferments malic in the same manner as fumaric acid—pyruvic acid being produced. Emmerling and Reiser [1902] have found fumaric acid among the products of fermentation of malic acid by *B. fluorescens*. In the case of muscle, it has been proved that fumaric acid is convertible to malic acid [Einbeck, 1919], the laevo-modification being formed [Dakin, 1922]. It would seem therefore that an equilibrium between fumaric acid and *l*-malic acid would be likely to occur with resting bacteria. Investigation showed that a solution of sodium fumarate in presence of resting *B. coli* gave rise to a solution with a laevo-rotation, and that sodium *l*-malate gave rise to fumaric acid. The crop of *B. coli* from three Roux bottles was added to 100 cc. of 1 % sodium fumarate and 20 cc. of p_H 7.2 buffer in a vacuum flask, which was evacuated and kept at 37° for 24 hours. The solution after filtration and concentration gave a laevo-rotation in presence of uranium acetate. A similar quantity of resting *B. coli* was incubated for 34 hours with 100 cc. of 1 % sodium *l*-malate and 20 cc. of p_H 7.2 buffer in an evacuated flask. The solution after this time showed a diminished laevo-rotation. It was filtered through kieselguhr and 50 mg. of fumaric acid were obtained by precipitation with silver from an aqueous solution of the crude acids obtained by acid ether extraction. The fumaric acid was identified in the same way as that obtained from succinic acid. In an open melting point tube it did not melt at 200° but showed signs of sublimation. Its melting point was 270° in a sealed melting point tube. 0.0050 g. dissolved in water required 0.68 cc. of 0.1136 *N* alkali for neutralisation (corresponding to 0.0049 g. fumaric acid). The aqueous solution was not optically active.

We have found only minute quantities of fumaric acid produced from malic acid by resting *B. coli*, the amounts depending on the activity of the

organism. That the laevo-variety of malic acid is preferentially attacked is shown by the fact that inactive malic acid yields a solution giving (with uranium acetate) a dextro rotation. It is clear that the rates of production of fumaric acid from malic acid and *vice versa* are extremely slow, even in presence of relatively large quantities of the organism. They are not comparable in order of magnitude with the velocities of these changes as produced by muscle tissue [Dakin, 1922]. On the other hand, the actual amount of the requisite enzyme in the resting organism in our experiments must be small compared with the amount present in the muscle used in Einbeck's or Dakin's experiments.

The slight inhibition by malic acid of the complete decolorisation of methylene blue by succinate in the presence of resting organism may possibly be ascribed to the production of a small quantity of fumarate.

Oxygen uptake.

It was of interest to ascertain whether oxygen was taken up by resting organism in presence of succinate and fumarate. The Barcroft differential apparatus was used and Tables II and III summarise the results. There is

Table II.

Absorption vessels	1	2	3
	1 cc. coli (10)	1 cc. coli (10)	1 cc. coli (10)
	1 cc. buffer 7·2	1 cc. buffer 7·2	1 cc. buffer 7·2
	1 cc. saline	1 cc. 1 % sodium succinate	1 cc. 1 % sodium fumarate
Time mins.	Oxygen absorbed (in cmm.)		
92	80·4	253·3	135·6
188	150·2	513·4	402·9
268	194·9	790·7	680·4

Temperature of bath = 35° C.

Table III.

Absorption vessels	1	2	3
	1 cc. <i>B. pyocyaneus</i>	1 cc. <i>B. pyocyaneus</i>	1 cc. <i>B. pyocyaneus</i>
	1 cc. buffer 7·2	1 cc. buffer 7·2	1 cc. buffer 7·2
	1 cc. saline	1 cc. 1 % sodium succinate	1 cc. 1 % sodium fumarate
Time mins.	Oxygen absorbed (in cmm.)		
70	369·7	458·2	515·9
103	571·6	823·4	948·0
164	855·7	1632·5	1926·2
209	1026·9	2319·8	2844·1

Temperature of bath = 35° C.

no doubt that both with *B. coli* and *B. pyocyaneus*, the absorption of oxygen by the resting organism is largely increased in the presence of either succinate or fumarate. This was to be expected from the results given in the previous paper [Quastel, 1924]. In spite of the fact that oxygen was taken up by sodium fumarate in presence of *B. pyocyaneus*, tests for the presence of pyruvic acid (the guaiacol and nitroprusside tests) proved completely negative. Now since pyruvic acid is undoubtedly formed by *B. pyocyaneus* growing in a fumarate medium, and since it is unlikely that the resting organism will have

a different mode of oxidising fumaric acid, it would seem that with the resting organism, pyruvic acid is more quickly dealt with than with the growing organism. This has yet to be proved, but it is reasonable to ascribe the failure to obtain evidence of the production of a ketonic acid by the resting organism to differences between the rates of oxidation of fumaric and pyruvic acids by the resting and growing organisms respectively. The non-appearance of a ketonic acid when *B. coli* is grown on a fumarate medium might thus be explained as due to differences between the rates of oxidation by growing *B. coli* and growing *B. pyocyaneus* and not to the absence in the former of a specific enzyme capable of producing the ketonic acid.

DISCUSSION.

Four main points emerge from these investigations. The first point is the association of the enzyme with the structure of the resting organism. It is apparent from the effects of hydrogen ion concentration and of temperature on the rates of decolorisation of methylene blue by succinate that a close connection exists between the chemical activity and the structure of the organism.

There is in the second place the close analogy between fresh muscle and resting bacteria (as represented by *B. coli* and *B. pyocyaneus*). The existence in these bacteria and in muscle of an enzyme capable of oxidising the saturated molecule of succinic acid to the unsaturated one of fumaric acid makes it probable that the enzyme is one of widespread occurrence. The reaction too is one of extreme importance, for it demonstrates the ability of the cell to produce a most reactive structure—the unsaturated linkage—from a saturated molecule. The next step apparently is the linking of oxygen on to the unsaturated bond to produce a ketonic structure—from fumaric acid, oxalacetic acid. It might be conceived that the mechanism of this oxidation of fumaric acid lies through the intermediate formation of malic acid, by the addition of the elements of water, and the subsequent giving up of two atoms of hydrogen. Thus:

This, however, is extremely unlikely, for malic acid is incapable of donating hydrogen to methylene blue in presence of resting organism. It would also be expected on the above hypothesis that fumaric acid would slowly reduce methylene blue in the presence of resting organism: this does not occur. Again fumaric acid has been found among the products of fermentation of malic acid [Emmerling and Reiser, 1902], but no malic acid has been found among the products of fermentation of fumaric acid [Quastel, 1924].

Such a connection between the related unsaturated and hydroxy acids, e.g. fumaric and malic acids, is of significance when we consider it in the light of the general association in nature of these types of acids. The possibility of an equilibrium, for instance, between crotonic and β -hydroxybutyric acids, is suggested, and experiments are being made to test this point.

We have found that a number of substances besides succinic acid are capable of transferring hydrogen to methylene blue in presence of resting organism, and our next communication, we hope, will deal with these.

The third point which emerges is the reversibility of the reaction between succinic acid, methylene blue, fumaric acid, and *leuco*-methylene blue in presence of the resting organism. We have found no evidence with the resting organism for Thunberg's theory [1920] that fumaric acid is a donator of hydrogen and exists in equilibrium with acetylenedicarboxylic acid. This may possibly occur in the case of muscle—so far as we know the matter has not yet been thoroughly investigated—but in the case of resting bacteria all the evidence points to fumaric acid being an acceptor of oxygen in the sense that it links oxygen to its molecule and is not a hydrogen donator.

This brings us to the fourth point, which is the distinction which must be drawn between methylene blue and oxygen as hydrogen acceptors. Fumaric acid can "accept" oxygen and yet not transfer any hydrogen to methylene blue. This makes it clear that, either there are two distinct modes of biological oxidation (apart from the rôle of glutathione), or else that the hypothesis [Wieland, 1913] by which an oxidisable substance must itself be capable of giving up hydrogen to a suitable acceptor must be modified.

It is a point worthy of further mention that though the change succinic acid $\rightarrow$ fumaric acid, is a good example of Wieland's view, this view would necessarily involve for the further oxidation of fumaric acid the addition of the elements of water to form malic acid and the donation by this acid of two atoms of hydrogen. We find no evidence under the conditions of our experiments for this procedure.

SUMMARY.

1. Resting bacteria (*B. coli* and *B. pyocyaneus*) possess an enzyme, similar to that existing in muscle, which is capable of bringing about the reduction of methylene blue in presence of sodium succinate.

2. The reversible equilibrium

succinic acid + methylene blue $\rightleftharpoons$ fumaric acid + *leuco*-methylene blue is set up in presence of the resting organism. The equilibrium constant of this equation has been obtained. Fumaric acid has been isolated from a succinic acid—methylene blue mixture.

3. The reversible equilibrium

appears to exist in presence of resting organism. Fumaric acid has been isolated in small quantity from *l*-malic acid after the action of resting organism.

4. The effects of hydrogen ion concentration and of temperature on the reduction times of methylene blue by succinate in the presence of organism have been investigated and attention is drawn to the close association between chemical activity and physical structure of the organism.

5. It has been shown that fumaric acid readily absorbs oxygen in presence of resting organism, though it will not donate hydrogen to methylene blue. This is discussed in relation to Wieland's hypothesis.

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