XLVII. SOME REACTIONS OF RESTING BACTERIA IN RELATION TO ANAEROBIC GROWTH.

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I. Anaerobic growth of facultative anaerobes.

The circumstances in which the facultative anaerobes will grow under strictly anaerobic conditions are far from clear. It is known that the presence of carbohydrates capable of attack by the organism in question favours anaerobic growth; Ritter [1907] has found that, besides carbohydrates, some of the higher alcohols and hydroxy acids are efficient. Nitrates have been found to induce anaerobic growth of facultative anaerobes [Ritter, 1907; Veillon and Mazé, 1910] and Beijerinck [1903] has perceived the possibility that the liberation of energy from nitrate and sulphate oxidations might be utilised by micro-organisms for growth. A mass of data has accumulated on the bacterial reduction of nitrates to nitrites since the discovery of the phenomenon by Schönbein in 1868. Maassen [1901] has shown that 85 out of 109 types of bacteria tested will bring about the reduction. It is generally accepted to-day that the biological significance of the reduction of nitrate is that by it oxygen is supplied to an organism when free oxygen is no longer available.

The facts, however, are somewhat disconnected and little systematic analysis of the conditions under which anaerobic growth of the facultative anaerobes occurs appears to have been attempted.

II. The action of resting bacteria on nitrates.

Haas and Hill [1923] showed that in milk there was a system which could bring about the reduction of nitrate to nitrite and Dixon and Thurlow [1924] have been able to demonstrate that nitrate will replace methylene blue in the Schardinger reaction and in the xanthine oxidase reaction in milk.

There can be little question that nitrate is capable of acting as a hydrogen acceptor in a manner similar to methylene blue.

What part, then, is played by nitrate when this is present in a bacterial medium? Does it remove hydrogen in the sense that sulphite removes acetaldehyde in Neuberg's fermentations, and thus divert the course of the

normal processes? Or does it play a more significant and positive rôle which would, for instance, be quite distinct from that of a fixative, or of methylene blue considered merely as a hydrogen acceptor?

Resting bacteria have been defined [Quastel and Whetham, 1924] as bacteria which have been grown for two days on tryptic broth, centrifuged, washed with saline and finally very well aerated. They are used under such conditions that multiplication of the cell does not occur. The reactions of the resting bacteria under our experimental conditions are thus due to the non-proliferating organism.

It was found that in the presence of resting bacteria succinic acid donates hydrogen to methylene blue with the production of fumaric acid. It was also found that in the presence of the bacteria fumaric acid oxidises leucomethylene blue, so that finally an equilibrium is established between succinic acid, fumaric acid, methylene blue and leucomethylene blue.

Now it is clear from the oxidising action of fumaric acid upon leucomethylene blue (which does not occur *in vitro*) that the resting bacteria activate the fumaric acid molecule. Activations of methylene blue and leucomethylene blue do not occur, for these are substances which are easily capable of accepting and donating hydrogen respectively *in vitro*, *i.e.* without activation. They form mixtures capable of giving definite oxidation-reduction potentials, measurable at an electrode, and are typical of normal oxidation-reduction systems of which the ferric-ferrous system is an example. Methylene blue and similar substances can therefore be regarded in a sense as already activated *in vitro*. Succinic acid, on the other hand, cannot donate hydrogen *in vitro*. Hence its power of reducing methylene blue *in vivo* must be ascribed to an activation of the acid by the cell. Similarly the oxidising action of fumaric acid *in vivo* must be attributed to an activation of this acid.

As in the case of muscle, there are many substances besides succinic acid which are donators of hydrogen to methylene blue in the presence of resting bacteria (*B. coli* or *B. pyocyaneus*). Among these are lactic acid, β-hydroxybutyric acid, glutamic acid, etc., and a detailed discussion of these will form the subject of a subsequent communication.

The influence of nitrate upon the rate of reduction of methylene blue by various hydrogen donators in presence of resting *B. coli* and resting *B. pyocyaneus* has been investigated. The experimental method was that employed in the work on succinic and fumaric acids [Quastel and Whetham, 1924].

Measurements of the velocity of reduction of methylene blue were made under anaerobic conditions in vacuum tubes at 45° in carefully buffered solutions at a p_H of 7·2.

The observations on methylene blue reductions recorded below are typical of the average of those made. The organism mainly employed for quantitative work was *B. coli comm.*, but *B. pyocyaneus* was sometimes used and found to give similar results.

The first experiments showed that nitrate has a most powerful inhibiting

influence upon the velocity of reduction of methylene blue in presence of the bacteria. (See Table I.)

Table I.

Tubes	(1)	(2)	(3)
cc. of emulsion of resting *B. coli*	1	1	1
cc. phosphate buffer solution p_H 7·2	1	1	1
cc. 1 % Na succinate solution	0·5	0·5	–
cc. 1 % K nitrate solution	–	1	–
cc. methylene blue solution 1/5000	1	1	1
	Reduced completely in 8 mins.	Remained deep blue.	Not completely decolorised in 2 hrs.

It was expected that since nitrate can act as a hydrogen acceptor in a manner similar to methylene blue it would prolong the time of reduction of the dyestuff through competition for the hydrogen. Unless, however, there was a preferential attack of the nitrate, this would be no explanation for the complete inhibition of the reduction of methylene blue. This inhibition resembled that due to fumarates and for further analysis of the phenomenon recourse was had to the technique employed in demonstrating the oxidising action of fumaric acid *in vivo.*

Use is made of an inverted U-tube which can be evacuated. In one limb of the tube is placed a mixture of resting organism, buffer solution, hydrogen donator solution, and methylene blue, and in the other limb a solution of the substance whose oxidising action is to be investigated. The tube is evacuated and, if necessary, filled with nitrogen. The methylene blue is allowed to become completely reduced at 45°, and the solutions in the two tubes are mixed. If there occurs a permanent blueing it is evident that an oxygen donator is present. To show that the oxidation is due to a thermolabile enzyme, the tube containing the reduced methylene blue is heated to any desired temperature before the solutions are mixed. If after heating there is no permanent blueing it is concluded that a thermolabile enzyme controls the oxidation. Blank experiments must of course be made to show that the substance in question does not oxidise leucomethylene blue *in vitro.*

By making use of this technique the following facts were established:

(1) Nitrate oxidises leucomethylene blue in presence of resting *B. coli* or *B. pyocyaneus*, but not *in vitro* (at p_H 7·2 and 45°). In equivalent concentration it is more powerful than fumarate in oxidising the leuco dyestuff.

(2) The oxidation by nitrate is dependent, like that by fumaric acid, on a thermolabile enzyme.

In one limb of a vacuum U-tube was placed a mixture of 1 cc. resting *B. coli,* 1 cc. 1 % sodium succinate solution, 1 cc. phosphate buffer solution, p_H 7·2, and 1 cc. 1/5000 methylene blue solution, and in the other 1 cc. of a 5 % sodium nitrate solution.

The technique was carried out as described above and no oxidation of the leucomethylene blue was found to occur; if the organism had not been heated the oxidation would have been extremely rapid.

(3) Nitrite cannot reduce methylene blue or oxidise leucomethylene blue in presence of resting *B. coli* or *in vitro* (at p_H 7·2 and 45°).

The enzyme dealing with nitrate, like that which deals with succinic acid and fumaric acid, appears to be closely associated with the physical structure of the organism. It is destroyed when the organism becomes coagulated either by heat or by treatment with acid.

These results suggest that when nitrate is added to a nutrient medium containing *B. coli* or *B. pyocyaneus* the nitrate around the cells becomes activated so that it is capable of oxidising substances in the vicinity of the cell (or at the cell surface) which it is incapable of oxidising *in vitro* (or in the body of the medium where the nitrate is outside the sphere of influence of the cell). Now there must be a definite pressure of nitrate molecules on every cell in the medium and since a number of these molecules become activated at the cell surface, each organism will become the centre of a sphere where active oxidations will proceed. Each cell may then be regarded as in a state comparable with that which exists under aerobic conditions.

In this manner the action of nitrate offers a distinct contrast to that of methylene blue, which does not require activation by the organism. Moreover, since the cell surface, like any enzyme, will become "saturated" with regard to the activated nitrate, the oxidation conditions around the cells, above a certain concentration of nitrate, will remain constant. Since nitrite is inert from the point of view of oxidation and reduction (*i.e.* by *B. coli*) and is an easily diffusible ion, its production will not affect the oxidation conditions, and hence the velocity of oxidation of a hydrogen donator at the cell surface. With methylene blue, however, the oxidation potential at the cell surface will depend on the relative proportion of methylene blue to leucomethylene blue and will vary as this proportion alters.

III. The action of resting bacteria on chlorates and perchlorates.

Using the same technique as with nitrate it was found that chlorate is a most powerful (anaerobic) oxygen donator. It will not oxidise leucomethylene blue *in vitro* but a vigorous oxidation occurs in the presence of resting *B. coli* or *B. pyocyaneus*. In the presence of chlorates complete reduction of methylene blue by a hydrogen donator *in vivo* does not appear to take place. Chlorate, moreover, will not effect the reduction of methylene blue by cysteine or glutathione (*i.e. in vitro* reduction); it will do so, however, in the presence of resting organism. As in the case of fumarates and nitrates, the action of chlorates depends on a thermolabile enzyme. (See Table II.)

Perchlorates neither oxidise leucomethylene blue *in vivo* nor *in vitro*. Bromates and iodates both oxidise leucomethylene blue *in vitro*.

IV. The action of resting *B. subtilis* on nitrates and chlorates.

Resting *B. subtilis* was investigated in a similar manner to *B. coli* and *B. pyocyaneus* and it was found that neither nitrates nor chlorates bring about

any perceptible oxidation of leucomethylene blue in presence of the organism. Fumarate oxidises leucomethylene blue in the presence of the organism only very slowly and certain strains do not appear to produce any oxidation. If *B. subtilis* can activate fumarate, nitrate or chlorate at all, the activation is very slight indeed.

Table II.

Tubes	(1)	(2)	(3)
cc. 1 % Na chlorate solution	1	1	1
cc. buffer solution p_H 7·2	1	1	1
cc. methylene blue solution 1/5000	1	1	1
cc. cysteine solution 4 mg./cc.	1	1	1
cc. resting *B. coli*	1	–	–
cc. resting *B. coli* which has been heated at 100° for 20 mins.	–	1	–
	Reduction of methylene blue never complete.	Rapid reduction in 10 mins.	Rapid reduction in 10 mins.

It is well known, of course, that *B. subtilis* will not bring about the reduction of nitrate to nitrite in a fermenting medium, whilst *B. coli* and *B. pyocyaneus* do so very readily.

V. Consideration of some conditions necessary for growth.

It is clear that for an organism to grow energy must be supplied to it for its synthetic operations. This is secured either by the oxidation of the substrate or by its anaerobic breakdown (occurring with the liberation of energy). It has been suggested [Stephenson and Whetham, 1924] that in the anaerobic growth of *B. coli* upon glucose the energy is derived from the anaerobic breakdown of glucose into lactic acid, a change involving the liberation of 22·56 Cal. per gram-mol. of glucose. It was shown, in fact, that even in the presence of air the early period of the growth of *B. coli* upon a glucose medium is anaerobic.

With lactic acid, succinic acid, glycerol, etc., all the likely paths of anaerobic breakdown appear to involve absorption of energy and on none of these compounds, where these formed the sole sources of carbon, and ammonia the sole source of nitrogen, was anaerobic growth of *B. coli* found to occur.

Beijerinck and Folpmers [1916] showed that fumaric acid and malic acid can be broken down by bacteria to pyruvic acid, and one of us [Quastel, 1924] was able to demonstrate that in the growth of *B. pyocyaneus* on ammonium fumarate, the whole of the oxygen absorbed during the first two or three days' growth was accounted for by oxidation of the fumaric acid to oxalacetic (or pyruvic) acid. Within the limits of experimental error no oxygen uptake by the bacteria alone could be demonstrated. In other words the molecular oxygen utilised simply oxidised the fumaric acid to pyruvic acid. The latter molecule entered into the organism's synthetic operations and the necessary energy for synthesis was derived from the fumaric acid oxidation. This point is worthy of emphasis for it clearly demonstrates that given (1) an adequate source of energy, (2) the appropriate molecule to enter into the organism's

synthetic reaction, the presence of molecular oxygen may not be necessary for growth.

There is another point to consider. If the organism is incapable of activating a substrate molecule, *i.e.* if it does not possess a mechanism which will induce the molecule to liberate energy (*e.g.* either by oxidation or by anaerobic breakdown), it is clear that this molecule cannot serve as a basis for a nutrient medium. For instance, if an organism cannot activate fumaric acid so that the latter may become oxidised so as to liberate energy and form pyruvic acid, this organism will not grow upon ammonium fumarate as sole source of carbon and nitrogen.

B. subtilis cannot activate fumaric acid to any appreciable extent (see above) and, as has been previously shown, it does not grow aerobically (or anaerobically) on ammonium fumarate. *B. coli* and *B. pyocyaneus* activate fumaric acid and both grow aerobically upon ammonium fumarate.

The following three conditions at least seem, therefore, to be necessary before growth may occur:

(1) The organism must be able to secure energy either by anaerobic decomposition of the substrate (*e.g.* glucose into lactic acid) or by the oxidation of the substrate.

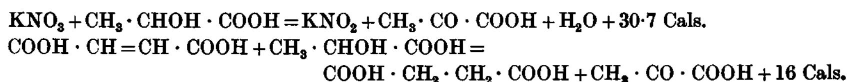
(2) The organism must be able to activate the substrate so that the latter is capable of reaction, *e.g.* oxidation or reduction, which it does not necessarily undergo *in vitro*.

(3) The products of oxidation (or decomposition) of the substrate, or the substrate itself, must be capable of entering into the synthetic operations of the organism.

It follows that if molecular oxygen is replaced by some molecule which induces around the bacterial cell a state comparable with that which occurs under aerobic conditions, and so long as the three conditions enumerated above are fulfilled, then anaerobic growth should be possible in presence of this molecule.

It has been shown above that we have reason to suppose that nitrate produces about the cell a state comparable with that existing under aerobic conditions. Chlorate and fumarate, which both oxidise leucomethylene blue in presence of resting *B. coli* and *B. pyocyaneus* should therefore have effects similar to those of nitrate. Provided, then, that conditions (1), (2) and (3) enumerated above are complied with, the presence of one of these substances (nitrate, chlorate or fumarate) should enable *B. coli* or *B. pyocyaneus* to grow anaerobically on compounds, such as lactic acid, which will not alone normally support anaerobic growth.

Condition (1) is satisfied by lactate and nitrate, and lactate and fumarate, as the following equations show:



$$KNO_3 + CH_3 \cdot CHOH \cdot COOH = KNO_2 + CH_3 \cdot CO \cdot COOH + H_2O + 30{\cdot}7 \text{ Cals.}$$

$$COOH \cdot CH{=}CH \cdot COOH + CH_3 \cdot CHOH \cdot COOH = COOH \cdot CH_2 \cdot CH_2 \cdot COOH + CH_3 \cdot CO \cdot COOH + 16 \text{ Cals.}$$

Condition (2) is satisfied as shown by the results described in section II.

Condition (3) is satisfied by the production of pyruvic acid.

Since perchlorate on the other hand is not activated by *B. coli* or by *B. pyocyaneus*, it cannot be expected to produce anaerobic growth or oxidation, and since nitrate is not apparently activated by *B. subtilis* it should not induce the anaerobic growth of this organism.

VI. Anaerobic growth in the presence of nitrates.

Throughout the following experiments the inorganic medium employed was as follows:

0·4 g. $(NH_4)_2HPO_4$.
0·1 g. NaCl.
0·1 g. KH_2PO_4.
0·07 g. $MgSO_27H_2O$.
100 cc. distilled water.

To this was added 1% of a carbon compound.

The solution was brought to a p_H of 7·2 and autoclaved in test-tubes in 10 cc. lots. Each of them was inoculated with 0·1 cc. of a 20-hour broth culture of *B. coli* or *B. pyocyaneus* and incubated aerobically or anaerobically as required. Experience has shown that it is very desirable to inoculate with relatively heavy sowings of the bacteria in order to obtain consistent and regular results.

The results are enumerated below.

A. with *B. coli*.

(1) Growth of *B. coli* occurs under strictly anaerobic conditions in the presence of nitrates, and with pyruvic acid, lactic acid, succinic acid, fumaric acid or glycerol as the sole source of carbon. No growth occurs anaerobically on these substances in the absence of nitrates.

(2) *B. coli* produces from lactic acid aerobically and anaerobically in the presence of nitrates a relatively large quantity of pyruvic acid. The yield is of the order obtained from *B. pyocyaneus* growing upon fumaric acid. *B. coli* when growing aerobically upon lactic acid in the absence of nitrates does not liberate pyruvic acid. This phenomenon will be discussed in more detail later.

(3) Growth of *B. coli* in the presence of nitrates is always accompanied by the production of large quantities of nitrite.

B. with *B. pyocyaneus*.

(1) Extensive growth of *B. pyocyaneus* occurs in the presence of nitrates under strictly anaerobic conditions on citric acid, glucose, lactic acid, or pyruvic acid as sole source of carbon. *B. pyocyaneus* will not grow anaerobically upon these substances (with the exception of glucose) in the absence of nitrates. It will grow anaerobically to some extent on glucose alone without nitrates, but not nearly to the same extent as with nitrates. *B. coli*, on the other hand, grows very well on glucose alone anaerobically and the growth is similar to that obtained when nitrates are present.

B. pyocyaneus both aerobically and anaerobically liberates pyruvic acid from lactic acid in the presence of nitrates. It will produce pyruvic acid from lactic acid aerobically in the absence of nitrates (difference from *B. coli*). The growth of *B. pyocyaneus* upon citric acid and nitrate anaerobically and upon citrate alone aerobically is extremely luxuriant. We have found it very convenient to use a tube of citrate medium (containing no nitrate) inoculated with *B. pyocyaneus* as a test for anaerobiosis. In the presence of a trace of oxygen growth will occur.

Good growth, but not so extensive as with citric acid, occurs anaerobically in the presence of nitrates with succinic acid, fumaric acid, or glycerol as sole source of carbon.

(2) *B. pyocyaneus* breaks down nitrites anaerobically. This is not the case with *B. coli*. (This was originally observed by Maassen [1901].)

C. with *B. subtilis*.

No anaerobic growth of this organism has yet been obtained. This agrees with the observation that *B. subtilis* is incapable of activating nitrate.

It is interesting to note that *B. coli* which will not produce pyruvic acid from lactic acid aerobically in the absence of nitrates, will do so in the presence of nitrates. It has been shown by Mazé [1918] that pyruvic acid is formed from lactic acid by several micro-organisms and we have noted the excellent yields of pyruvic acid produced by *B. pyocyaneus* in a well-aerated medium. In the work on fumaric acid it was shown that *B. pyocyaneus*, but not *B. coli*, will liberate pyruvic acid. It was questioned whether this difference between the two organisms was due to the absence from *B. coli* of a specific enzyme present in *B. pyocyaneus*. Later evidence was brought to show [Quastel and Whetham, 1924] that "the non-appearance of a ketonic acid when *B. coli* is grown on a fumarate medium might be explained as due to differences between the rates of oxidation by growing *B. coli* and growing *B. pyocyaneus* and not to the absence from the former of a specific enzyme capable of producing the ketonic acid." This view is clearly corroborated by the nitrate experiments described above. It also agrees with Mazé's observations on the production of pyruvic acid from lactic acid.

VII. The effect of resting *B. coli* on lactate and nitrate: isolation of pyruvic acid.

20 cc. of a thick suspension of resting *B. coli* were placed in a vacuum tube capable of holding 100 cc. To this were added 10 cc. of a neutral 1 % sodium lactate solution, 10 cc. of a 5 % sodium nitrate solution and 10 cc. of a phosphate buffer solution, p_H 7·2. The tube was evacuated and placed in a thermostat at 45° for three hours. The contents of the tube were then centrifuged and the clear supernatant solution treated with phosphoric acid and warmed. Urea was added to remove all nitrite. After cooling, a freshly-prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid was added, and the *p*-nitrophenylhydrazone of pyruvic acid was separated and identified.

VIII. The effect of resting *B. coli* on lactate and chlorate: production of chlorite and pyruvic acid.

The same procedure was adopted as in the above experiment, using chlorate solution instead of nitrate.

After three hours at 45° the presence of pyruvic acid could be demonstrated by the nitroprusside reaction. It was isolated as the *p*-nitrophenylhydrazone. Some chlorate had been reduced to chlorite. The presence of chlorite could be demonstrated by adding a little potassium iodide to the solution, a little starch solution and finally dilute acetic acid. There was an immediate liberation of iodine. Hypochlorite was not formed since this would have reacted with potassium iodide in neutral or alkaline solutions. The chlorite can be estimated iodometrically in the presence of dilute acetic acid.

The oxidising effect of chlorate upon lactate in presence of resting *B. coli* is more vigorous than that of nitrate for equivalent concentrations. No oxidation of lactate by either nitrate or chlorate occurs *in vitro*.

IX. The course of the production of nitrite and pyruvic acid during the growth of *B. coli* on lactate and nitrate.

B. coli was grown aerobically and anaerobically in an inorganic medium containing 1 % lactic acid as sodium lactate, and 1 % sodium nitrate, and the production of nitrite and pyruvic acid was followed in both conditions.

The tubes were inoculated as usual and incubated aerobically and anaerobically. They were examined in duplicate after varying intervals of time. The nitrite produced was estimated iodometrically. 2 cc. of the medium were placed in a small wash bottle, a little potassium iodide and starch solution were added and nitrogen bubbled through vigorously. Dilute sulphuric acid was added and the iodine liberated was estimated by titration with $N/10$ thiosulphate solution.

The pyruvic acid production was followed by the nitroprusside reaction. To 2 cc. of the medium were added solid ammonium sulphate, a few crystals of sodium nitroprusside and finally 2 cc. of strong ammonia. In the tables below a single plus sign indicates a positive but feeble reaction, whilst a number of plus signs indicates an intense reaction demonstrating the production of a large quantity of pyruvic acid. Table III gives the results of one aerobic series and Table IV gives the results of a parallel aerobic and anaerobic series. The maximum amount of sodium nitrite possible from 1 % sodium nitrate is 0·81 %.

It is generally found that the pyruvic acid liberation by *B. coli* growing in a medium containing 1 % lactate and 1 % nitrate is irregular and feeble until the nitrite produced reaches a concentration equivalent approximately to 0·4 % sodium nitrite. After this concentration is reached the pyruvic acid liberation is increased enormously, whilst the actual amount of growth does not appear to increase.

Table III.

Hours from time of inoculation	Growth	Pyruvic acid produced (examined by nitro-prusside reaction)	p_H	% sodium nitrite produced
15	+ + +	+ +	6·8	0·45
20¾	+ + +	+ + + +	6·8	0·57
31¾	+ + +	+ + + + +	7·1	0·65
41½	+ + +	+ + + + +	7·25	0·76
48	+ + +	+ + + + + (0·3 % by colorimetric comparison)	7·3	0·79
65*	+ + +	+ + + + +	7·4	0·81

* An examination of these tubes showed that practically all the cells present were dead.

Table IV.

Hours from time of inoculation	Growth		Pyruvic acid (indicated by nitroprusside reaction)		% sodium nitrite produced	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
18	+ +	+ +	+	+	0·33	0·34
23	+ + +	+ + +	+	+	0·38	0·38
41½	+ + +	+ + +	+ + + + +	+ + + + +	0·69	0·72
66	+ + +	+ + +	+ + + + +	+ + + + +	0·76	0·77

Moreover this course of events occurs both in aerobic and anaerobic conditions. In other words *B. coli* grows on a mixture of lactate and nitrate in the same way whether the conditions are aerobic or anaerobic.

Pyruvic acid was isolated after approximately 50 hours' growth in the following way. To each tube a little chalk was added and the mixture well shaken and filtered. The filtrate, which was practically free from protein matter, was treated with syrupy phosphoric acid (1 cc. of acid for each tube), excess urea was added, the solution warmed and finally well aerated. When all the nitrite was decomposed, the solution was refiltered, cooled, and treated with a little freshly-prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid. The precipitated hydrazone was filtered through a Buchner funnel, washed with glacial acetic acid and water and recrystallised from dilute alcohol.

It was identified as the *p*-nitrophenylhydrazone of pyruvic acid by its melting point (219°) and mixed melting point.

X. The toxic effect of nitrite upon *B. coli*.

To a series of tubes containing 1 % lactate and 1 % nitrate was added sodium nitrite in varying quantities. These tubes were inoculated with *B. coli* and the growth and amount of nitrite present after 16 hours' incubation were examined in the usual way. The following table gives the results.

Table V.

% nitrite present initially	Growth after 16 hours		% nitrite present after 16 hours		% nitrite produced	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0	+ + +	+ + +	0·45	0·45	0·45	0·45
0·01	+ + +	+ + +	0·30	0·32	0·29	0·31
0·05	+ +	+ +	0·27	0·28	0·22	0·23
0·15	+	+	0·20	0·19	0·05	0·04
0·35	0	0	0·36	0·36	0·01	0·01
0·6	0	0	0·6	0·6	0·0	0·0

The concentration of sodium nitrite toxic to *B. coli* in a 1 % lactate 1 % nitrate medium appears to be about 0·35 %. Although this strength of nitrite causes cessation of growth of *B. coli* it does not prevent resting *B. coli* from activating nitrate and lactate, so that the latter interact and produce nitrite and pyruvic acid. This is illustrated by the following experiment. A series of tubes containing 1 % lactate, 1 % nitrate and 0·4 % nitrite was prepared. To each tube was added 1 cc. of resting *B. coli*. After 20 hours each tube showed an increase in the quantity of nitrite present and pyruvic acid was formed as indicated by the positive nitroprusside reaction. This phenomenon affords an explanation of the fact that much pyruvic acid does not appear during the growth of *B. coli* on a lactate-nitrate medium until the nitrite production has reached about 0·4 %. Below this strength of nitrite the pyruvic acid produced from the lactate is almost entirely used for growth. As soon, however, as the nitrite reaches its toxic concentration and prevents further bacterial synthesis, the pyruvic acid, which is still being produced from the lactic acid by the action of nitrate and the resting organism, is diverted from synthesis and is liberated into the medium.

XI. The breakdown of nitrite by *B. pyocyaneus*.

The breakdown of nitrite by *B. pyocyaneus* is a well-known phenomenon. The following experiment shows the ease with which it is effected.

A medium containing 1 % lactate and 0·1 % nitrite and another containing 1 % lactate and 0·05 % nitrite were sterilised and sown with 0·1 cc. of a 20-hour broth culture of *B. pyocyaneus* and incubated aerobically. The following table gives the results.

Table VI.

Hours after time of inoculation	0·05 % nitrite (as K salt) medium	0·1 % nitrite (as K salt) medium
21½	All nitrite utilised	0·03 % nitrite still present
41½	—	All nitrite utilised

XII. Aerobic and anaerobic growth of *B. coli* in the presence of chlorates.

So far it has been found impossible to substitute chlorates for nitrates in anaerobic growth, although the organism has been shown to be capable of activating chlorate. This failure of chlorate to induce anaerobic growth is probably due to the production of chlorite (p. 312), which is toxic to *B. coli* at a concentration of 1 in 20,000.

The following series of experiments (see Table VII) shows the toxic effect of chlorate on *B. coli* growing on lactate and nitrate.

Tubes containing 1 % lactate, 1 % nitrate and varying quantities of potassium chlorate were inoculated in the usual way and incubated at 37° both aerobically and anaerobically.

Table VII.

% $KClO_3$	0·5	0·1	0·025	0·005	0·001	0
Aerobic.						
Growth after 16 hours from time of inoculation	0	0	? +	+ + +	+ + +	+ + +
Growth after 44 hours from time of inoculation	+	+	+	+ + +	+ + +	+ + +
Pyruvic acid production (indicated by nitroprusside reaction)	+ +	+ +	+ + + +	+ + + +	+ + + +	+ + +
Anaerobic.						
Growth after 44 hours from time of inoculation	0	0	+	+ + +	+ + +	+ + +
Pyruvic acid production (indicated by nitroprusside reaction)	0	0	0	+ + + +	+ + + +	+ + +

The concentration of potassium chlorate, which is completely toxic to *B. coli* anaerobically under these conditions, is about 0·03 % or 1 in 3000. Aerobically there is a definitely toxic effect of chlorate at about this concentration, but there is slight growth at concentrations of chlorate much higher than 0·03 %.

XIII. The toxic effect of chlorites.

Potassium chlorite was prepared according to the method of Bruni and Levi [1915]. The chlorite obtained was estimated iodometrically (in the presence of dilute acetic acid) and the solution made up with distilled water to 1 %. It was brought to p_H 7·2. The solution was not autoclaved since considerable loss of chlorite is occasioned by so doing—moreover, it is unnecessary since chlorite is a most powerful antiseptic.

Tubes containing 1 % lactate 1 % nitrate medium were prepared and sterilised; to these were added varying quantities of chlorite and the tubes were inoculated with *B. coli*. They were incubated aerobically and anaerobically. Table VIII gives the results.

Table VIII.

% $KClO_2$	0·07	0·05	0·03	0·01	0·005	0
Aerobic.						
Growth after 24 hours	0	0	0	0	(+)	+ + +
,, ,, 48 ,,	0	0	0	0	(+)	+ + +
Anaerobic.						
Growth after 48 hours	0	0	0	0	(+)	+ + +

The fact that resting *B. coli* has been shown to produce chlorite from chlorate and the fact that chlorite is toxic at extremely low concentrations explains why chlorate is ineffective as a substitute for nitrate in anaerobic growth.

XIV. Aerobic and anaerobic growth of *B. coli* in the presence of perchlorates and of maleic acid.

Since perchlorate and maleate [Quastel and Whetham, 1924] do not appear to be activated by resting *B. coli* it was expected (1) that perchlorates and maleates would not act as substitutes for nitrates in anaerobic growth; (2) that perchlorates and maleates would not be toxic to *B. coli* growing upon lactate and nitrate.

Experiment shows that neither perchlorates nor maleates will induce anaerobic growth and that they have no perceptible toxic action on *B. coli* growing upon lactate and nitrate, under aerobic or anaerobic conditions.

XV. Aerobic and anaerobic growth in the presence of fumarate.

If the general conceptions which have been outlined above are correct it should follow that for anaerobic growth nitrates may be replaced by some organic hydrogen acceptor or oxygen donator, so long as this substance is activated by the organism and there is the necessary liberation of energy, etc.

Fumaric acid was chosen as a suitable hydrogen acceptor and experiment showed that *B. coli* will grow anaerobically (in the absence of nitrates) on a mixture of lactate and fumarate where these substances form the sole sources of carbon and ammonia the sole source of nitrogen. On lactate alone and on fumarate alone no anaerobic growth of *B. coli* occurs. For experiment the following solutions were made up:

A. 1 % lactic acid as Na salt, 1 % fumaric acid as Na salt, and the usual inorganic medium.

B. The same with 2 % fumaric acid.

These were autoclaved, inoculated as usual, and incubated aerobically and anaerobically. The results are indicated in Table IX.

Table IX.

	1 % lactate, 1 % fumarate medium	1 % lactate, 2 % fumarate medium
Aerobic.		
Growth after 48 hours	—	Very good throughout medium
p_H after 48 hours	—	7·0
Growth after 72 hours	Very good throughout medium	Very good throughout medium
p_H after 72 hours	7·6	7·0
Anaerobic.		
Growth after 48 hours	—	Very good throughout medium
p_H after 48 hours	—	6·8
Growth after 72 hours	Very good throughout medium	Very good throughout medium
p_H after 72 hours	6·8	6·8

When *B. coli* grows aerobically on fumarate or anaerobically on a mixture of fumarate and nitrate the solution becomes alkaline ($p_H > 7·4$) owing to the decarboxylation of the oxidised fumaric acid. Since lactic acid is a monobasic acid the growth of *B. coli* upon this does not produce immediate alkalinity,

but a slight acidity owing to the ammonia which is utilised being more than equivalent to the alkali set free by the utilisation of the lactic acid. Hence, if in a mixture of lactic and fumaric acids there is produced immediate alkalinity after growth of *B. coli*, this would show that fumaric acid is being utilised; if an acidity, this would be evidence for the utilisation of lactic and the non-utilisation of fumaric acid. In all the anaerobic growths of *B. coli* on mixtures of lactic and fumaric acids there is produced slight acidity indicating the utilisation of lactic acid and that the fumaric acid is acting solely as hydrogen acceptor. This was further confirmed by the isolation of succinic acid from these media.

For experiment, twenty tubes, each containing 10 cc. of a 1 % lactate, 1·25 % fumarate medium, were inoculated with *B. coli* and incubated anaerobically for 12 days. All tubes showed good growth; the p_H of the solution was 6·9. The contents of the tubes were mixed and 20 cc. of syrupy phosphoric acid were added. The acid solution was extracted with ether and the white residue from the ether extract dissolved in a little water, silver nitrate solution added, the mixture filtered, and the clear filtrate just neutralised with sodium hydroxide. There was a copious precipitate, which, on decomposition by sulphuretted hydrogen, yielded a solution which did not reduce permanganate and gave a crystalline residue of succinic acid, identified by the melting point (185°) and by a mixed melting point.

It is specially noteworthy that fumaric acid can play a dual rôle:

(1) It will act as an oxygen acceptor and become a nutrient carbon source, *e.g.* in the anaerobic growth of *B. coli* on fumarate and nitrate.

(2) It will act as a hydrogen acceptor and so allow lactic acid (for instance) to be utilised, *e.g.* in the anaerobic growth of *B. coli* on a mixture of fumarate and lactate.

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