166. *The Growth of Coliform Bacteria in Media containing Nitrate and Nitrite. Part II. Influence of Ammonia and of Aeration, and the Coupling of the Oxidation–Reduction Systems involved.*

By P. R. Lewis and C. N. Hinshelwood.

With lowered oxygen supply the reduction of nitrate by *Bact. lactis aerogenes* increases. Sudden aeration of an anaerobically growing culture leads to a catastrophic drop in the rates of reduction of nitrate and nitrite.

Growth in ammonium salts inhibits nitrate and nitrite reduction. When the ammonia is used up the reduction process begins again, but only after a period of recovery, longer for nitrate. The delayed recovery seems to be due to an inhibitor which is removed during growth.

These and other results are interpreted in terms of a kinetic scheme (shown in Table I) according to which the normal dehydrogenase mechanisms of the cell involve an oxidation–reduction system, X,XH_2, which also participates in other processes. The ratio $[XH_2]/[X]$ expresses and determines the reducing potential prevailing in the cell. Aeration lowers this ratio and impedes nitrate and nitrite reduction. Optimum growth in ammonia demands a low value of the ratio (as shown by the need for good aeration) : thus optimum growth in ammonia and optimum reduction of nitrate or nitrite are incompatible.

(1) *Introduction.*—In Part I (previous paper) there has been occasion to refer to the coupling of nitrate reduction mechanisms with the normal dehydrogenase activity of the cells. First, in the absence of their carbon source the bacteria will not reduce nitrate or nitrite, and, secondly, training of certain strains of *Bact. coli* to grow in a glucose–ammonium sulphate medium confers *pari passu* increased adaptation to nitrate.

This idea comes into still greater prominence in a further series of studies now to be described, which are conveniently discussed before the experiments on the later stages of reduction of nitrite, since the results of the former are important for the interpretation of the latter.

(2) *Influence of Aeration Conditions on Reduction of Nitrate and Nitrite.*—In view of the probable importance of the normal dehydrogenase systems in determining the rate of reduction of nitrate and nitrite, a study was made of the dependence of these rates upon the degree of aeration of the test media. Stickland (*Biochem. J.*, 1931, **25**, 1543) observed that some strains of *Bact. coli* reduced nitrate to nitrite (which was apparently not further used by the cells) more rapidly under anaerobic conditions, the nitrate ion providing the oxygen necessary for the general metabolism of the cells.

First, therefore, the accumulation of nitrite during the growth of *Bact. lactis aerogenes* in the nitrate test medium was measured under various conditions of aeration. There seemed little advantage in making a series of experiments at controlled partial pressures of oxygen, since the rate of accumulation of nitrite varies in any case during growth, and quantitative interpretation

FIG. 1.

Rate of nitrite accumulation and degree of æration.

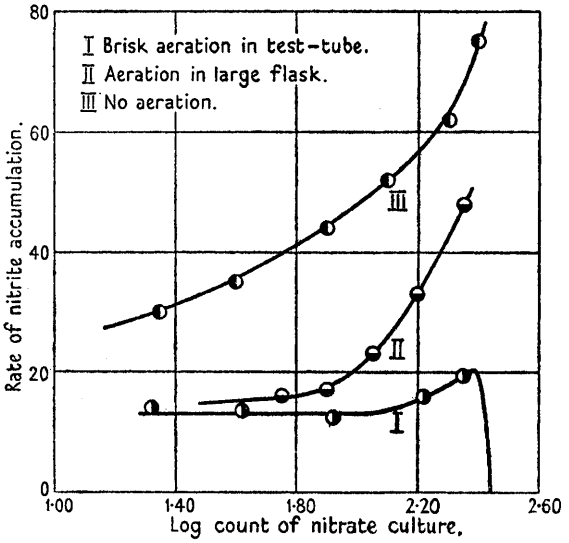
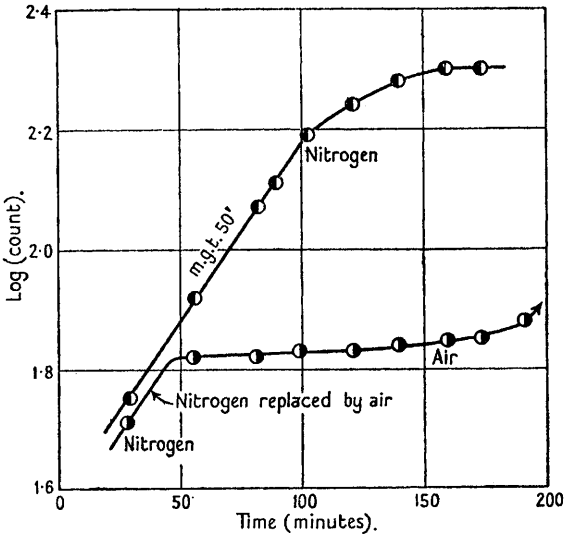


FIG. 2.

Growth of (nitrate-trained) Bact. lactis aerogenes *in nitrate medium, with discontinuous æration changes.*



of the results would be difficult. [The steady rise in the rate of accumulation during growth is particularly noticeable under conditions of comparatively poor aeration (Fig. 1), and itself presents an interesting problem. Either the amount of the enzyme system which reduces nitrate to nitrite increases during growth or else its efficiency increases, the latter alternative being the more likely.]

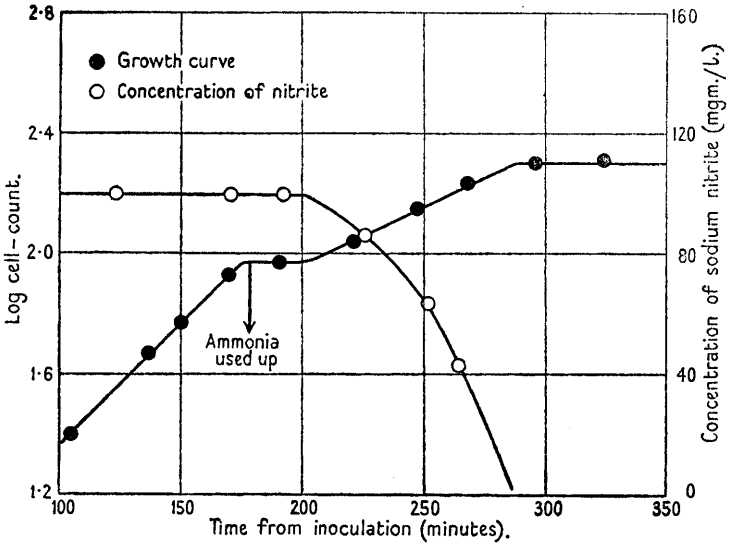
The most informative experiment was carried out as follows. A nitrate test medium was

inoculated and kept saturated with a stream of nitrogen. When logarithmic growth was well established and a small amount of nitrite had accumulated (Fig. 2), the nitrogen was replaced by air. Within a short time all the nitrite disappeared and growth stopped completely. It did not restart for more than two hours, by which time about 10 mg./l. of nitrite had accumulated. Another nitrate medium (inoculated in parallel) which was kept saturated with nitrogen throughout behaved quite normally. Thus the sudden increase in the partial pressure of oxygen leads to a catastrophic drop in the rates of reduction of nitrate and nitrite.

In explanation of these results it may be assumed that some cell metabolite, which will be denoted XH_2, is readily oxidised, either by the oxygen of the air or by other suitable oxidising agents, those present competing for the available hydrogen. A fall in the concentration of a more powerful oxidising agent will tend to allow an increase in the concentration of XH_2 to the point where the rate of reduction of others becomes appreciable. Thus if the concentration of oxygen is reduced, the rate of reduction of nitrate may well increase, as is actually found. More than one readily oxidisable metabolite and several enzyme systems may well be involved in the transfer of hydrogen, but these considerations do not fundamentally affect the situation, provided that the various systems are in a balanced condition, as they almost certainly are during logarithmic growth.

FIG. 3.

Growth and nitrite consumption in mixed ammonium sulphate–nitrite medium.



The upward trend of the rate of nitrate reduction during growth is understandable. When the count becomes large the rate of oxygen consumption may exceed the rate of supply to the medium by solution of the gas. There will then be a drop in the rate of hydrogen transfer with a consequent accumulation of oxidisable metabolites and an increase in nitrate reduction. The count which has to be exceeded before this occurs will be higher the more efficient the degree of aeration. That this is indeed so is shown in Fig. 1 which gives the rate of nitrate accumulation as a function of n for a series of experiments carried out with differing degrees of aeration.

That the rate of nitrite reduction is not affected in this way by restricted aeration may be inferred from the fact that the growth rate, which appears to be determined by the rate of nitrite reduction, was the same in all the above experiments.

Under anaerobic conditions the inhibitory action of nitrite on growth, already referred to, becomes much more pronounced. This effect almost certainly accounts for the lower total population in nitrate media which are inadequately aerated. An early falling off of the growth rate was observed in the enzyme experiments previously described and was accompanied by a sharp increase in the rate of nitrate reduction to a value of 200—250. The oxidisable metabolites apparently begin to accumulate rapidly as the nitrite inhibition depresses the growth rate. Sometimes there is a subsequent fall in the rate of nitrate reduction, due presumably to exhaustion of the reserves of oxidisable metabolites. Under highly aerobic conditions (cf. Part I, Fig. 3) high concentrations of nitrite do not accumulate and logarithmic growth is

brought to an end by a drop in the rate of nitrate reduction, caused presumably by the failure of the supply of oxidisable metabolites.

High concentrations of nitrite were found to reduce the growth rate in ammonium sulphate as well as in the nitrite medium itself. When, in a nitrate medium, the reduction in the growth rate by the accumulation of nitrite occurs there is a sharp rise in the rate of nitrate reduction. This suggests that just as growth is stopping the oxidisable compounds postulated above are, as it were, thrown on the market and give the extra burst of nitrate reduction.

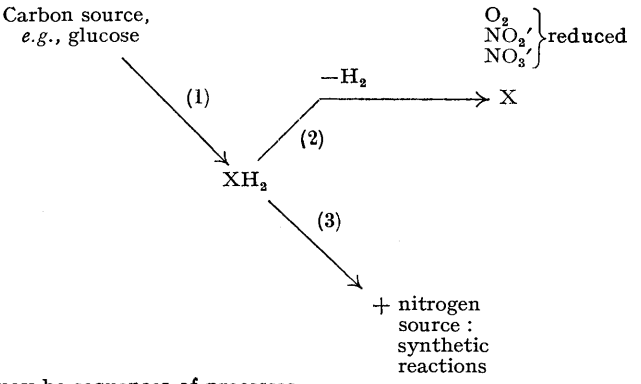
If we postulate the scheme given below, the interpretation of various results can be summarised as follows :

(*a*) Lack of oxygen leads to enhanced reduction of nitrate since [XH_2] rises.

(*b*) Inhibition of growth by excess nitrite lowers the rate at which XH_2 is removed by process (3) and increases [XH_2] : hence the rate of reduction of nitrate rises.

(*c*) With a plentiful supply of oxygen accumulation of toxic products interferes first with (1) and this leads to a drop in [XH_2] and thus in rate of nitrate reduction.

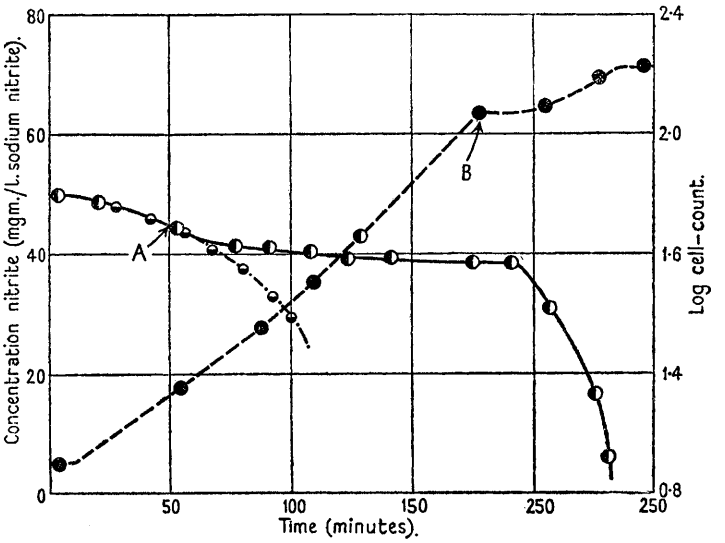
This scheme is considered further in Section 4.



(1), (2), and (3) may be sequences of processes.

FIG. 4.

Effect of addition of ammonium sulphate on the removal of nitrite from a growing nitrite culture.



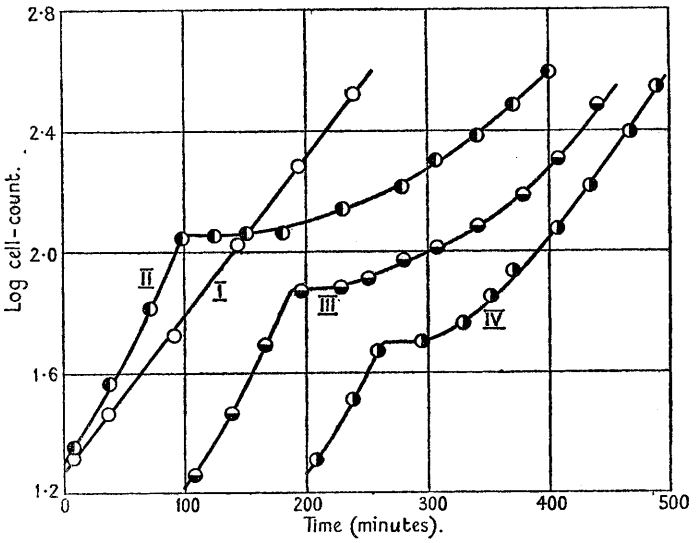
● Growth curve.
◐ Concentration of sodium nitrite, actual (mgm./L.).
⊖ Concentration of sodium nitrite, calculated on the assumption that the rate of utilisation is unaffected by addition of ammonia.

The ammonium sulphate is added at the point A, *and has become exhausted at the point* B.

(3) *Influence of Ammonia on the Reduction of Nitrite and Nitrate by* Bact. lactis aerogenes.—The addition of ammonia to a medium inoculated with the normal strain of *Bact. lactis aerogenes* leads to an almost complete inhibition of nitrite removal, which does not restart until the amount of ammonia remaining is negligible. If the addition is made before growth has started, little or no nitrite is removed (Fig. 3) : if it occurs subsequently the rate of nitrite removal falls rapidly within 10 minutes and eventually reaches zero (Fig. 4). The utilisation of nitrite restarts 10—15 minutes after the concentration of ammonia has become quite insignificant. The actual amount of ammonia present at this stage was determined indirectly by a separate experiment which showed that the amount present 10 minutes after rapid growth has stoppod corresponds to less than 1 mg./l. of ammonium sulphate (limit of detection).

FIG. 5.

Effect of adding ammonium sulphate to actively growing cultures in the nitrate medium.



Curve I. No ammonia added.
Curve II. 100 mgm./l. ammonium sulphate added at time = 0.
Curve III. 60 mgm./l. added at time = 100′.
Curve IV. 30 mgm./l. added at time = 200′.

Repeated subculture in the nitrate medium does not bring about any detectable change in the rate at which the nitrite utilisation is slowed up after the addition, but with the trained strain the removal of nitrite restarts while there is still some ammonia present (Fig. 6*a*). [In some experiments the removal of nitrite was again temporarily halted (for about 15—25 minutes) just as the last appreciable traces of ammonia disappeared from the medium, a complication which is not relevant to the main conclusions.]

The addition of ammonia to an actively growing nitrate culture likewise causes a rapid fall in the rate of nitrate reduction. When all the ammonia has been used up, the growth rate drops for a time to zero and subsequently rises again to its optimum value (Fig. 5).

The time, θ, taken for the growth rate to recover to half its optimum (in nitrate), once ammonia utilisation has stopped, is fairly reproducible. Some values obtained are given in the Table, which shows that the value of θ is roughly proportional to the amount of ammonia added, provided that the addition is made during the early stages of logarithmic growth. If the ammonia is added in the later stages of growth its effect is much less.

Log (initial count).	Δ $(NH_4)_2SO_4$ (mg./l.).	θ (mins.).	θ/Δ $(NH_4)_2SO_4$.	Log (initial count).	Δ $(NH_4)_2SO_4$ (mg./l.).	θ (mins.).	θ/Δ $(NH_4)_2SO_4$.
ca. 0·8	80	200	2·5	1·30	100	240	2·4
ca. 0·8	80	215	2·7	1·43	30	35	1·2
1·15	60	175	2·9	*ca.* 1·45	80	80	1·0
1·22	60	165	2·7	1·50	80	65	0·8
1·27	30	70	2·3	1·62	80	45	0·6

Imperfect aeration reduces the inhibitory action of ammonia on the nitrate reduction process, thus allowing a concentration of nitrite to be built up. If, when all the ammonia has been used up, there is some nitrite as well as nitrate present in the culture medium, growth in the nitrite restarts almost immediately, and within 15 minutes nitrate reduction has returned almost to the optimum rate.

The delayed recovery suggested that, during growth in ammonia, some substance is formed which inhibits the reduction of nitrate to nitrite. If this were so, dilution of the culture by inoculation into a new medium once all the ammonia has been removed should reduce the length of the recovery period. A series of nitrate media were therefore inoculated at intervals from a parent nitrate culture to which ammonia had been added and the lags in these media determined. In every case, the lag was less than 30 minutes, whereas the length of the recovery period in the parent medium was about 200 minutes. Thus dilution of the growth medium had in fact enabled the nitrate-reduction process to recover to its optimum rate in a much shorter time. It was relevant to find out what effect filtrate from cells grown in ammonium sulphate has upon cells growing in nitrate. Definite evidence on this point proved difficult to obtain since there was usually still some ammonia remaining in the filtrate. In one or two experiments addition of filtrate did have a greater retarding effect than could reasonably be accounted for by the residual ammonia, but in most other experiments the result was inconclusive. Addition of filtrate containing no ammonia did not cause a definite arrest, but merely a slight slowing down of growth.

The inhibitory substance formed during growth in ammonia, when added in the form of filtrate, can apparently be removed very rapidly during growth in nitrate. This suggests that it is a normal metabolite used during growth in both nitrate and ammonia.

Experiments with a nitrate-trained strain of *Bact. lactis aerogenes* gave results essentially similar to the above, though the process of recovery after the period of arrest exhibited some additional complications. Three media containing the following nitrogen sources, were inoculated with a nitrate-trained strain: (i) 1000 mg./l. of sodium nitrate; (ii) 50 mg./l. of sodium nitrite; (iii) 1000 mg./l. of sodium nitrate and 50 mg./l. of sodium nitrite.

Once logarithmic growth had definitely started, 100 mg./l. of ammonium sulphate was added to each medium and growth followed; the concentration of nitrite present in the last two media was also determined at intervals. The results are shown in Fig. 6. In medium (iii) the concentration of nitrite began to fall about 10 minutes after the ammonia had been added, showing that the production of nitrite was slower than its rate of removal [curve III (*BC*)]. The nitrite concentration finally became steady (*CD*): during the corresponding period of time (*C'D'*), the concentration of nitrite in medium (ii) was also stationary. Hence the reduction of both nitrite and nitrate had ceased. At point *D* removal of nitrite started again, but from *E* to *F* the nitrite concentration was once more stationary. The same happened in medium (ii) showing that nitrite removal is temporarily stopped, whilst the rate of nitrate reduction is still very low. Rapid logarithmic growth in the ammonia ceased just after the point *E*. At point *F* growth restarted, with consequent removal of nitrite, and the rate of nitrate reduction slowly increased so that the concentration of nitrite reached an almost steady value (*HJ*) until the end of logarithmic growth. Here again the substance which inhibits the nitrate reduction process seems to be removed very rapidly during growth in nitrite.

To recapitulate the essential facts: growth in ammonia inhibits nitrate and nitrite reduction. When the ammonia is used up, the reduction processes begin again, but only after a period of recovery, longer for nitrate. The delayed recovery seems to be due to an inhibitor which is removed during subsequent growth. The recovery process may show certain other complexities, not investigated further at the moment.

(4) *Discussion.*—The facts described in Section 3 may now be brought into relation with those of Section 2.

We have seen that the reduction of nitrate and of nitrite by the organisms studied in these experiments appears to be closely linked with the normal dehydrogenase activity of the cell. In the simplest and most general form this idea is expressed by the diagram in Section 2.

XH_2 is formed from the carbon source, and, in the ordinary course of aerobic growth is oxidised (directly or indirectly) by molecular oxygen. This skeleton scheme, which represents in essentials the kinetics of a great many detailed possibilities, leads to some interesting results.

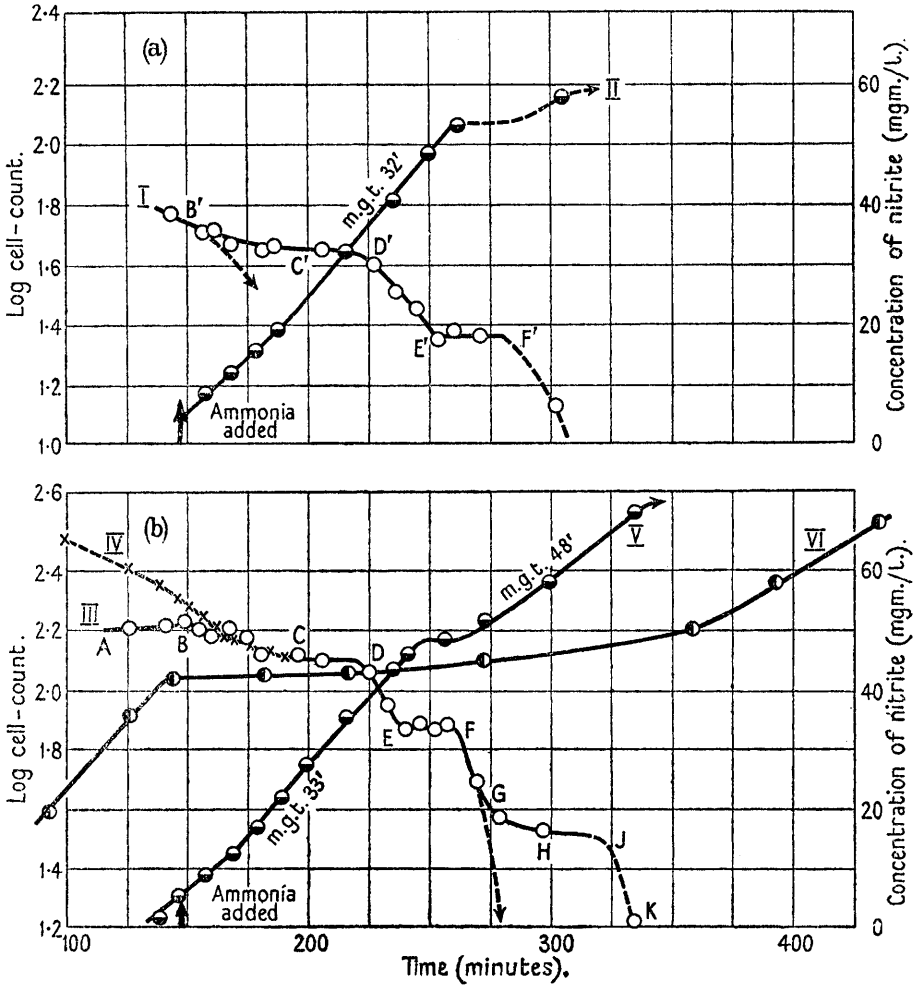
The ratio of [X] to [XH_2] will be determined by the oxidation–reduction balance prevailing in the cell. It will express the oxidation–reduction balance which other conditions impose, and it will actually determine the possibility of the occurrence of specified oxidations or reductions while these conditions exist.

If we were dealing with reversible redox equilibria, there would be an oxidising potential proportional to $\ln[X]/[XH_2]$ (or to an analogous expression). Even when there is not true reversibility the oxidation–reduction relationships will show a general parallelism to that indicated by the hypothetical redox potential. It will be convenient in the following discussion

FIG. 6.

Effect of added ammonium sulphate on the nitrate and nitrite reduction processes of a nitrate-trained strain.

(a) *Effect on nitrite consumption. Medium (ii), nitrite only, inoculated at* t = 0 : 100 *mg./l. of ammonium sulphate added at* t = 145 (*about* 100 *minutes after start of growth*).
(b) *Effect on nitrate reduction. Medium (i), nitrate only, with ammonium sulphate addition. Medium (iii), nitrate plus nitrite. Ammonium sulphate added as in* (a).



(a) *Curve I, nitrite concentration. Curve II, growth curve in medium (ii).*
(b) *Curve III, nitrite concentration in medium (iii). Curve IV, nitrite concentration calculated on assumption that no nitrite is formed from nitrate. Curve V, growth curve in medium (iii). Curve VI, growth curve in medium (i) for comparison (ammonium sulphate added at* t = 0).

to speak in terms of the redox potential which would express the prevailing condition in the cell : but this will not have any direct implication about measurable electrochemical magnitudes.

In normal aerobic growth of *Bact. lactis aerogenes* with ammonium salts as a source of nitrogen we know that, in the above sense, conditions correspond to a relatively high (oxidising) potential. This is shown by the experimental fact that lack of aeration impedes growth and causes a sharp reduction in the final bacterial population (Lodge and Hinshelwood, *J.*, 1943, 208). Thus we

may infer that the ratio $[X]/[XH_2]$ for the steady state prevailing during logarithmic growth in ammonia is high, and in these conditions nitrate and nitrite cannot be reduced. This is the plain interpretation of the fact that growth in ammonia inhibits the reduction of nitrate and the utilisation of nitrite.

When ammonia is not being utilised, but the dehydrogenation mechanisms continue, $[XH_2]$ increases and the potential becomes more reducing until reduction of nitrate and nitrite becomes possible once more. In these terms the inhibition of nitrate and nitrite utilisation by ammonia and the subsequent recovery are generally understandable. The recovery process itself is further discussed below.

The $[X]/[XH_2]$ ratio which is optimal for the reducing stage of nitrate utilisation is not compatible with that which is optimal for ammonia utilisation. If ammonia is an intermediate in the further reduction of nitrite then a compromise must be established. A steady state must be set up in logarithmic growth such that the rate of formation of ammonia is equal to its rate of consumption. Here the concentration of the ammonia would have to be low but finite : if it rose higher it would inhibit nitrate reduction; if it dropped lower it would be insufficient to maintain the steady growth rate. This condition is not compatible with optimum growth in ammonia, and we find that the mean generation time is about 50 minutes for *Bact. lactis aerogenes* in nitrate, compared with 33 minutes for the same strain in an ammonium salt medium. (Ammonia itself may not in fact be an intermediate in nitrite utilisation, and indeed the oxidising potential required for growth in ammonia may indicate that oxidation occurs to a step intermediate between ammonia and nitrite. This matter is further considered in Part III, following paper.)

When cultures are well aerated, the re-oxidation of XH_2 is easy and the reducing potential drops, so that the reduction of nitrate and nitrite occurs less readily than in the poorly aerated cultures. Restriction of the oxygen supply lowers the redox potential and has two linked effects—nitrate reduction is enhanced, but ammonia utilisation is impaired. This corresponds to the experimental facts set forth and discussed in Section 2.

Another curious observation, described in Part I, probably finds its explanation in similar terms. Training of *Bact. coli* by serial subculture in a glucose–ammonium sulphate medium confers training to nitrate. Now it is known that there is a rather close parallelism between dehydrogenase activity and the growth of the coliform organisms in these media. The training process develops the dehydrogenases, and with their increased efficiency the concentration of XH_2 required for the reduction of nitrate is more readily built up. Hence the training to utilise nitrate.

Although it is not directly connected with the experimental work of this paper, another important phenomenon may be mentioned here, because it can very probably be interpreted in terms of an almost exactly analogous kinetic scheme. This is the Pasteur effect, in which aerobic growth with cell oxidation inhibits the competing process of fermentative breakdown.

It was mentioned above that recovery from the inhibition of nitrate and nitrite utilisation caused by addition of ammonia does not occur immediately the ammonia is used up, but sets in gradually, and at a rate which is a rather complex function of the conditions. There is in general a sort of induction period during which growth at the expense of nitrate or nitrite is resumed in an autocatalytic, and at first extremely slow, manner. The nitrite-utilising function recovers before the nitrate-reducing function. One might, at first sight, have expected a rapid change in the $[X]/[XH_2]$ ratio on addition of the ammonia, and a correspondingly rapid shift when the ammonia is used up, the transitory equilibrium values of the various intermediate concentrations of the reaction sequence being established with considerable rapidity. The effect of ammonia, on addition, does in fact declare itself fairly rapidly, but the re-establishment of the original state after removal of the ammonia is slow. This means clearly that during the growth in ammonia there is built up a reserve of substance which buffers the oxidising potential to a value unsuitable for nitrite or nitrate reduction. Only as this substance is removed in the course of further cell growth can the potential drop successively to the values required for reduction of nitrite and nitrate respectively. Since actual growth is required for the removal, the long autocatalytic period is easily understandable. The above interpretation also explains why the recovery is facilitated by dilution of the culture, why it takes longer, other things being equal, in proportion to the integral amount of chemical action ($\int n dt$) which has occurred in presence of ammonia, and, possibly, why it is subject to other more complex influences in the later stages of growth.

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