67. *The Growth of Coliform Bacteria in Media containing Nitrate and Nitrite. Part III. The Later Stages of Reduction of Nitrite.*

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During growth of *Bact. lactis aerogenes* in phosphate–glucose media containing nitrate the steady concentration of ammonia formed is about 1 mg./l., which is of the order to be expected (from the quantitative relations of various growth rates) if the nitrogen utilisation occurs by way of ammonia. The results are, however, not numerically precise enough to provide positive evidence for this view.

High concentrations of nitrite inhibit growth, but the action is not due to a reversal of nitrite reduction or to simple competition between nitrite and ammonia for sites on enzyme surfaces.

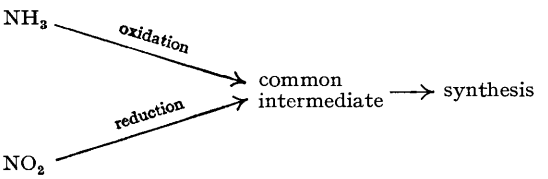
Hydroxylamine at quite low concentrations (from about 4 mg./l.) also inhibits growth, and if it is an intermediate in nitrite reduction cannot accumulate in amounts greater than a few parts per million. There is no direct proof but a little indirect evidence for its formation in very small quantities. Its inhibitory action is transient in that it is converted into another compound which, however, is neither nitrite nor ammonia.

(1) *Introduction.*—Nitrate is reduced to nitrite by the coliform bacteria used in these experiments (Parts I and II, previous papers), the accumulation and subsequent disappearance of nitrite being easily measurable.

The utilisation of nitrite by the cells almost certainly depends upon its further reduction, possibly by way of hydroxylamine to ammonia. Experiments have therefore been made with the object of obtaining evidence bearing upon the later stages of the reduction.

The formation of ammonia as an intermediate, though likely enough on purely chemical grounds, is by no means necessary. Efficient utilisation of ammonium salts by coliform bacteria is dependent upon adequate aeration. The possibility of an initial oxidation of ammonia must therefore be taken into account, and the following two routes considered :

$$NO_3' \longrightarrow NO_2' \longrightarrow NH_2{\cdot}OH \longrightarrow NH_3 \longrightarrow \text{synthesis}$$



The common intermediate might or might not be hydroxylamine. Hydroxylamine has been suggested as the member of the nitrate reduction series which combines with substances of the glycolysis cycle to give amino-acids. Thus Nord and Mull (*Adv. Enzymology*, 1945, **5**, 165) have postulated that, with Fusaria, hydroxylamine, formed by the reduction of nitrite, combines with pyruvic acid to give the oxime, which is further reduced to alanine.*

(2) *Experimental Methods.*—These were in general as described in Part I, except that micro-determinations of ammonia and of hydroxylamine were required. The method adopted for the former was that described by Conway and O'Malley (*Biochem. J.*, 1942, **36**, 655). This involves micro-distillation in special shallow dishes divided into two compartments. Approximately 1 ml. of a borate buffer containing screened indicator (a mixture of bromocresol-green and methyl-red) is pipetted into the inner compartment, and a 1 ml. or 2 ml. of test sample into the outer; 1 ml. of a saturated solution of potassium metaborate is then added, the lid of the dish adjusted, and the closed unit rocked gently to mix the sample and the alkali. The distillation of the ammonia into the inner compartment is 99% complete within 2—4 hours, but samples were normally left overnight. The units are then opened and titrated with N/70-sulphuric acid, added from a calibrated microburette to the inner compartment, until the indicator shows a faint pink tinge.

Numerous controls and duplicate experiments are carried out. After use the units are washed several times under running warm water and distilled water and dried at 110°; no alkali or acid is used for washing.

It was found possible to determine about 5 mg./l. of ammonium sulphate with 10% accuracy. With higher concentrations the accuracy was proportionately better, the probable error in most determinations being about ± 2%. By taking numerous samples, concentrations down to 1—0·5 mg./l. could be determined though not with accuracy.

The only practicable method for the determination of hydroxylamine was that based upon oxidation by N/10-iodine in glacial acetic acid to nitrite which was then determined by the method described in

* It has also been shown that in plants a little hydroxylamine is present during utilisation of nitrates, and that under physiological conditions it will react with various ketonic compounds to give oximes—the reaction with oxalacetic acid being especially rapid. This leads, after reduction, to aspartic acid, the principal free amino-acid found in plants if there is an adequate supply of a nitrogen source (Virtanen and Laine, *Nature*, 1938, **141**, 748).

Part I, after removal of excess of iodine by N/10-sodium thiosulphate. Even this method was not, quantitatively, wholly satisfactory.

(3) *Ammonia Formation during Growth in Nitrite.*—The first matter to be investigated was the formation of ammonia in the nitrate or nitrite media.

The following series of experiments were made. First, determinations were made by Conway's method of the concentration of ammonia prevailing at various stages during the growth of *Bact. lactis aerogenes* in the standard nitrate and nitrite media (Parts I and II). The amounts found, although detectable, were too small to be measurable with much precision, being of the order of 1 part per million of the medium, or less. Some typical results are recorded in Table I.

TABLE I.

pH.	Cell count.	mg./l.	pH.	Cell count.	mg./l.
5·52	80	0·0—0·4	6·80	50	0·0—0·3
5·35	110	0·1—0·8	6·72	105	0·3—1·2
5·50	50	0·0—0·6	6·60	200	0·3—1·2
5·40	60	0·1—0·5	6·40	390	0·2—1·0

If the sequence is: nitrate ⟶ nitrite ⟶ ammonia ⟶ synthetic reactions, then in the steady state the rate of production of ammonia will equal its rate of utilisation. Now the optimum overall growth rate in nitrite corresponds to a mean generation time of 50 minutes; the optimum growth rate in an ammonium salt medium corresponds to one of 33 minutes. If ammonia production and consumption are just balanced, it would follow that at a steady concentration of 1 part per million the mean generation time in this medium should have fallen to about 50 minutes. The relation between ammonia concentration and rate has not hitherto been determined, the only available results showing that the fall from the optimum does not occur until the concentration drops to a very small value. Experiments were made to determine the ammonia concentration at which the mean generation time would rise to 50 minutes, the method used being that of Dagley and Hinshelwood (*J.*, 1938, 1930). The value proved to be extremely low, once again of the order of 1 part per million, and it could not be determined more accurately (Table II).

TABLE II.

Concentration of ammonium salts necessary to maintain a growth rate of 2/3 optimal under various conditions of pH.

pH.	$[(NH_4)_2SO_4]$ (mg./l.).	pH.	$[(NH_4)_2SO_4]$ (mg./l.).
6·9	*ca.* 0·6	5·5	<0·5
6·9	<1·0	5·5	*ca.* 1·2
6·1	*ca.* 1·2	5·5	<1·5
6·1	<0·7	5·5	*ca.* 0·5—1·5 (or less)

It may be said, therefore, that the results are compatible with the view that the rate of production of ammonia balances the rate at which it could be independently consumed at the steady concentration prevailing during growth in nitrate, but that at such low concentrations the measurements cannot be made accurately enough to provide positive proof of this view.

(4) *Inhibition of Growth by Nitrite.*—Sodium nitrite at concentrations from 100 to 1000 mg./l. has an increasing inhibitory effect upon the growth rate, whether in nitrite alone or with ammonia as an alternative nitrogen source (Table III). In this range of concentrations the nitrite has no effect on the lag or on the total population. (At higher concentrations there is a catastrophic drop in the latter from the normal value of about 1000 to 10, reached at 2 g./l.)

TABLE III.

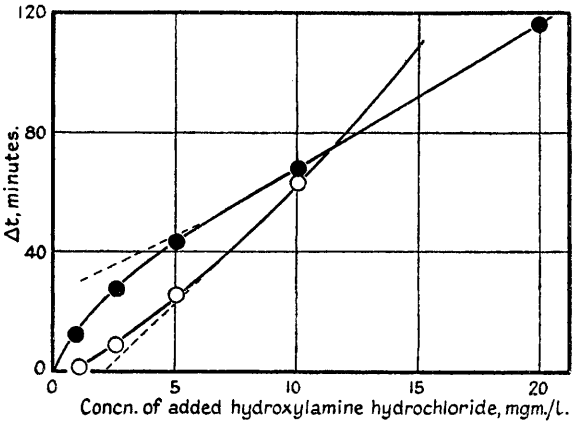
Inhibitory action of sodium nitrite.

	Mean generation time (mins.).			Mean generation time (mins.).	
Concn. of nitrite, g./l.	Ammonium salt medium.	Nitrate medium.	Concn. of nitrite, g./l.	Ammonium salt medium.	Nitrate medium.
0	32	52	0·6	52	—
0·1	35	54	0·8	64	80
0·2	36	55	1·0	—	95
0·4	42	64			

The fact that nitrite reduces the growth-rate in ammonia is suggestive, for it is the converse of the effect whereby the reduction of nitrite at low concentrations is inhibited by the presence of

ammonia. At first sight these two effects might seem to be alternative manifestations of some general phenomenon, such as competition between nitrite and ammonia for sites on the same enzyme surface. Conversion of ammonia into protein presumably occurs at some surface upon which ammonia is adsorbed, possibly in the form of an active radical such as > NH which then combines with various " carbon fragments ". The sites which adsorb ammonia might also adsorb nitrite ions which are then reduced to the same intermediate radical as is formed by oxidation of ammonia. Addition of ammonia to a growing nitrite culture could then cause a decrease in the rate of nitrite utilisation, and conversely the addition of excess of nitrite could reduce the rate of ammonia utilisation. Excess of nitrite slows down its own reduction and utilisation, so that at high concentrations it would have to displace the > NH radicals from the surface where they were formed before they could be used in synthesis. These displaced radicals would presumably form ammonia, detectable amounts of which should therefore accumulate during growth in high concentration of nitrite. Experiments were made to test for such accumulation, but none could be detected. The competition hypothesis is therefore untenable, at least in its simple form.

Delay produced in actively growing cultures by addition of hydroxylamine.



Open circles—ammonium sulphate media.
Black circles—nitrate media.

(*The final slopes of the lines are not comparable since the initial counts were different in the two sets of experiment.*)

A second possibility is that the enzyme which brings about the reduction of nitrate to nitrite also catalyses the reverse reaction. Sufficiently high concentrations of nitrite might then tend to set up a reducing potential within the cell, and so impair growth. If nitrite does in fact act in this way, its inhibitory effect should be reversed by high concentrations of nitrate. An experimental test was made. Under aerobic conditions even a five-fold excess of nitrate did not effect the growth rate, and under anaerobic conditions nitrate had a slight beneficial effect.

The balance of evidence is therefore against the view that the inhibitory action of nitrite is due either to a competition between it and ammonia, or to a change of the redox potential by its tendency to act as a reducing agent (though it still seems remarkable that the two intermediates, nitrite and hydroxylamine, which are easily capable both of oxidation and reduction, act as inhibitors at appropriate concentrations, while nitrate and ammonia do not). A less elegant and more specific explanation must apparently be sought. In this connection the observations of Nord and Mull (*loc. cit.*) on Fusaria are of interest. Though potassium nitrite is a good source of nitrogen for Fusaria, high concentrations retard growth. Pyruvic acid accumulates as a result of inhibition of the enzyme responsible for its decarboxylation. The explanation suggested was that nitrous acid reacts with certain amino-groups necessary for normal metabolism. An analogous theory applicable to *Bact. lactis aerogenes* seems worth consideration. Inhibition of pyruvate decarboxylation would considerably reduce the efficiency of anaerobic glycolysis so that nitrite inhibition should be more pronounced in the absence of oxygen; addition of nitrate would supply some oxygen and thereby offset the inhibition.

(5) *The Rôle of Hydroxylamine.*—Hydroxylamine proved to be a strong inhibitor of growth. When media containing hydroxylamine and either nitrate, nitrite, or ammonia are inoculated,

there is a long lag, though, once division starts, growth rapidly reaches the optimum rate characteristic of the main nitrogen source (Table IV). The addition of hydroxylamine to a growing culture causes an immediate and complete inhibition which lasts for several hours, and is followed by a rapid recovery to the optimal rate. Nitrite removal and nitrate reduction are completely stopped.

TABLE IV.

Variation of lag of washed cells with concentration of hydroxylamine initially present.

Concentration (c) of NH_2·OH,HCl (mg./l.).	Lag, L (mins.).	$(L - 64)/c$.
10	152	8·8
16	210	9·1
24	265	8·4
40	417	8·9
60	590	8·8

Analysis showed that during this period of inhibition hydroxylamine slowly disappears from the medium, and that when growth restarts the residual concentration is low, though sometimes detectable. The length of the arrest is roughly proportional to the amount of hydroxylamine added and is less for ammonium sulphate than for nitrate or nitrite. During the arrest hydroxylamine is therefore presumably being removed at a constant rate. The rate of removal is not, however, directly proportional to the cell count. The actual relation (Table V) can be explained by assuming that under the experimental conditions the hydroxylamine is not only removed by the cells but also reacts slowly with the glucose to give a non-toxic oxime, the rate of this reaction being independent of cell count. If hydroxylamine is added to a complete medium some considerable time before inoculation, the lag produced is in fact shortened.

TABLE V.

Dependence of length of arrest upon cell count.

(Concentration of hydroxylamine hydrochloride added was 40 mg./l.).

Cell count.	Arrest (mins.).	Arrest × cell count × 10^{-3}.	Cell count.	Arrest (mins.).	Arrest × cell count × 10^{-3}.
61	200	12·2	27	360	9·7
47	260	12·2	20	400	8·0
46	280	12·9	15	460	6·9
41	310	12·7	6	900	5·4

If the only process by which hydroxylamine can be removed were that involving the bacteria, the figures in the last column should be constant. The progressive falling off below a count of 40 shows that hydroxylamine is being removed by some other process which is not dependent upon the cells.

The three most likely ways in which the hydroxylamine could be metabolised by the cells are (i) reduction to ammonia, (ii) oxidation to nitrite, (iii) condensation with some carbonyl compound to give an oxime. The reduction to ammonia was first investigated in some detail.

In the first experiments about 40 mg./l. of hydroxylamine hydrochloride was added to a growing culture containing a small amount of ammonia, the concentration of which was determined at intervals. During the period of inhibition the ammonia concentration remained unchanged: when rapid growth restarted the ammonia disappeared, the increase in bacterial mass being equal to that calculated on the assumption that only the ammonia had been utilised. During the next 24 hours there was a further very gradual increase in bacterial mass, presumably owing to a slow utilisation of the compound formed from the hydroxylamine, the bulk of the latter being sometimes eventually utilised for nitrogen synthesis (Table VI).

TABLE VI.

The utilisation of hydroxylamine as a nitrogen source.

Washed cells used for inoculum. Δn of 1·5 is approximately equivalent to 1 mg./l. NH_2·OH,HCl.

Conc. of NH_2·OH,HCl (mg./l.).	Initial count.	Count after 3 days.	Δn.	Percentage utilisation.
20	45	68	23	80
40	42	83	41	70
40	45	58	13	22
50	37	89	52	70
60	45	83	38	44
80	21	48	27	23

In another experiment a medium containing hydroxylamine as the sole nitrogen source was inoculated with washed cells. No appreciable amounts of ammonia or of nitrite could be detected at any stage, and growth was very slow, the mean generation time being about 1000 minutes. Several experiments of this type were carried out and the percentage of hydroxylamine finally utilised was calculated; it varied between 50 and 100%, being highest for low initial concentrations.

The results of all these experiments suggest that the bacteria convert hydroxylamine into some non-toxic compound which can be utilised as a nitrogen source, but only at a slow rate: this compound is neither nitrite nor ammonia, and may be an oxime formed by condensation with some ketonic intermediate of the glycolysis cycle.

These conclusions do not rule out hydroxylamine as a normal intermediate in the utilisation of either nitrite or ammonia. It does, however, follow that the concentration obtained in the steady state cannot exceed the very small value at which the inhibitory action would become evident. Special experiments were made to determine the threshold of this action, and it appeared that up to about 1·5—2 mg./l. would be possible as a steady concentration during growth in nitrate. The analytical method, however, is not sensitive enough to show whether any is in fact present.

The following observations yield some indirect evidence on this. The time required to attain a given bacterial count was determined for a series of cultures in the nitrate and in the ammonia medium with additions of various small amounts of hydroxylamine. The delay, ΔT, relative to a control experiment, was plotted against the concentration, with the results shown in the figure. For the ammonia medium there is a much less than linear rise up to about 4 mg./l. of hydroxylamine hydrochloride, showing that small amounts can be in fact be tolerated. For the nitrate media the effect is reversed, small amounts producing a proportionately greater inhibition. There is no sign of the initial tolerance as found for the ammonia medium, and this suggests, though it does not prove, that in nitrate-growth hydroxylamine may already be present up to the limit of what is supportable without inhibition.

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