The Biochemistry of the Nitrifying Organisms

5. NITRITE OXIDATION BY *NITROBACTER**

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Nitrobacter is an autotrophic soil bacterium that derives its entire energy supply from the reaction

Apart from the early studies of Meyerhof (1916*a, b*), there has been little biochemical work done on this organism since its discovery and isolation by Winogradsky (1890, 1891). The present paper records investigations into the mechanism of nitrite oxidation by *Nitrobacter*. Preliminary notes on some of our results have already appeared (Lees & Simpson, 1955*a, b*).

MATERIALS AND METHODS

Growth of the organisms. *Nitrobacter* was obtained from an Aberdeen garden soil by serial subculture in a medium made up as follows. To 10 l. of water were added 30 g. of KH_2PO_4 , 1 g. of CaSO_4 , 0.5 g. of MgCl_2 , 0.01 g. of MnCl_2 , 0.2 ml. of dialysed iron (B.P.). By addition of 10*M*-NaOH, the pH was adjusted to 7.8, which is the optimum pH for the strain of *Nitrobacter* used (Lees, 1954). The solution was filtered and, after addition of the appropriate amount of NaNO_3 , sterilized at 120° for 20 min. This medium (medium G) was used also for the propagation of the stock cultures which provided inocula for large-scale batch cultures. For large-scale batch cultures 100 ml. of the carbonate-phosphate suspension, used by Lees (1952) for the culture of *Nitrosomonas*, was added to each 10 l. of medium G before sterilization. Batch cultures were used for preparing suspensions of *Nitrobacter* cells suitable for biochemical study; the carbonate-phosphate suspension was added to facilitate the separation of these cells after growth. *Nitrobacter*, unlike *Nitrosomonas*, does not require a solid surface for growth, but *Nitrobacter* cells did adhere in some measure to the solid particles in the suspension and, in any case, were entrained by them when the cells were separated from the bulk of the medium by centrifuging.

The cells were grown at 28°. Stock cultures were carried in conical flasks; the depth of the medium was always less than 1 cm., to ensure adequate aeration. Batch cultures were grown in 10 l. of medium in 10 l. bottles, each fitted with a glass sparger through which air was pumped at about 10 l./min. The air was sterilized by passing it through 0.1*M*- CuSO_4 in $\text{N-H}_2\text{SO}_4$, then through a sterilized cotton-wool plug. A fresh stock, or batch, culture was initiated by inoculating the sterilized culture medium (containing 70 µg. of N/ml., as NaNO_3) with about 1% of its volume of stock culture. Samples of the newly inoculated culture were

taken each day and analysed for nitrite. After all the nitrite initially added had been oxidized, twice the original amount was added. After this had been oxidized, four times the original amount was added, and so on. Nitrite oxidation in both stock and batch cultures usually ceased after about 3 weeks, when a total of about 1.4 mg. of nitrite N/ml. had been oxidized. Since washed suspensions of *Nitrobacter* cells prepared from batch cultures that had oxidized 1.4 mg. of nitrite N/ml. were capable of oxidizing nitrite vigorously in the Warburg apparatus, the inability of cultures to oxidize more than 1.4 mg. of nitrite N/ml. was presumably connected with the accumulation of nitrate or other metabolic end-products or both in the culture medium.

Separation of cells from batch cultures. After a batch culture had oxidized about 1 mg. of nitrite N/ml., the *Nitrobacter* cells were reaped as follows. (i) The culture was centrifuged in a Sharples Centrifuge (Sharples Centrifuges Ltd., Stroud, Glos.). The liquid passing through the centrifuge was discarded and the solid, containing all the cells, was suspended in 100 ml. of water. (ii) The suspension was agitated for 3 min. in a rotating-blade household homogenizer to detach the cells from the solid particles. (iii) The suspension was then made to about 300 ml. and centrifuged for 10 min. at 700 *g*. This treatment precipitated the solid particles, but left the cells in suspension. The supernatant fluid was poured off and the solid particles were again agitated and centrifuged in the same way. (iv) The combined supernatant fluids from the two centrifugings were then centrifuged at 11 000 *g* for 10 min.; this treatment precipitated the cells as a compact yellow-brown mass. The cells were washed once with water and stored at 0° in the buffer solution used almost universally throughout this work, i.e. 0.03*M*- KH_2PO_4 brought to pH 7.8 by addition of 10*M*-NaOH. The yield of cells from 10 l. of batch culture was about 200 mg. dry wt.

Purity of the *Nitrobacter* cultures. The cultures used were not pure, but were always contaminated by a single heterotrophic organism (apparently a pink yeast) that presumably grew on organic compounds made by *Nitrobacter*. Numerous attempts to purify the cultures failed. These attempts included plating the cultures on to nitrite-agar and on to nitrite-silica-gel; colonies of *Nitrobacter* that grew on these media always showed the presence of the heterotroph when transferred to nutrient agar on which the heterotroph, but not the *Nitrobacter*, would grow. Attempts to purify the *Nitrobacter* by dilution, or by treatment of the cultures with tetracycline, an antibiotic to which *Nitrobacter* seems to be largely insensitive, were equally unsuccessful. We thus had to use impure cultures of *Nitrobacter* for our work. The degree of contamination of the cultures was measured in two different ways. (1) The ratio of *Nitrobacter* cells to heterotrophic cells in washed-cell suspensions was estimated by

* Part 4: Hofman & Lees (1953).

direct microscopical count; the *Nitrobacter* cells were distinguished from the heterotrophic cells partly by the greater resistance of the *Nitrobacter* cells to staining (Gibbs, 1919), partly by their smaller size. (2) The number of *Nitrobacter* cells in a culture that had oxidized a given amount of nitrite was calculated on the twin assumptions that a *Nitrobacter* cell had a dry wt. of about 10^{-13} g. and that the free-energy efficiency of *Nitrobacter* was about 5% (Baas-Becking & Parks, 1927); the number of heterotrophs in the washed-cell preparation of *Nitrobacter* from the same culture was measured by plating the preparation on to nutrient agar. According to both methods of measurement, the washed-cell preparations of *Nitrobacter* from batch cultures, used throughout the present work, never contained more than 1 heterotrophic cell : 50 *Nitrobacter* cells. It therefore seemed reasonable to assume that these preparations were suitable for investigations of the biochemical aspects of nitrite oxidation.

Measurement of nitrite oxidation. This was usually followed manometrically in the Warburg apparatus at 25°. Owing to the fact that the Q_{O_2} (μ l. of O_2 /mg. dry wt. of cells/hr.) for *Nitrobacter* in the presence of nitrite was 100–150, only about 3 mg. dry wt. of cells was required for each vessel. The cells were suspended in 2.5 ml. of 0.03M phosphate buffer, pH 7.8, and placed in the main vessel. Additions of $NaNO_2$ were made in 0.5 ml. of solution from the side arm some 10 min. later, after temperature equilibration of the Warburg vessels. In a few experiments nitrite oxidation was followed by measurements of the amount of nitrite that disappeared when a solution of $NaNO_2$ was incubated with *Nitrobacter*. Nitrite was estimated by a Griess–Ilosva technique (Lees & Quastel, 1946). Checks were also run from time to time on the amount of nitrate produced when a known amount of nitrite had been oxidized. These checks were based on a colorimetric estimation of nitrate by phenol-disulphonic acid (Lees, 1952).

Detection of cytochrome bands and measurement of their optical densities. The absorption bands of reduced cytochromes in *Nitrobacter* suspensions were detected, and their wavelengths roughly measured, by means of a Hartridge reversion spectroscope. The wavelengths and optical densities of the bands were precisely determined in a spectrophotometer. Difference spectra of the reduced and oxidized cytochrome components of *Nitrobacter* were obtained by comparing, at regular wavelength intervals, the optical density of a washed, well-aerated *Nitrobacter* suspension with that of a similar suspension treated with dithionite. The spectroscopic data for Fig. 8 were obtained by measuring the optical density of the *Nitrobacter* suspensions reduced by dithionite at 551 and 589 $m\mu$.; for all measurements the same sheet of white paper, having a white-light transmission roughly equal to that of the cell suspensions, was used as a 'blank'.

The cytochrome most intimately concerned with nitrite oxidation in *Nitrobacter* was apparently a cytochrome that, in its reduced state, showed an absorption band at 551 $m\mu$. (see 'Results'). This cytochrome will be referred to as 'cytochrome 551' in general; its reduced form will be designated $Fe_{cyt. 551}^{2+}$, and its oxidized form $Fe_{cyt. 551}^{3+}$.

Pre-incubation. In some experiments, *Nitrobacter* cells were pre-incubated with various compounds for different periods before being studied in the Warburg apparatus. The cells were suspended in 25 ml. of 0.03M phosphate buffer, pH 7.8, containing, where appropriate, the compound

under investigation. The pre-incubations were carried out at 25° in 100 ml. stoppered conical flasks; the flasks were usually shaken during incubation. From time to time a sample of 1–3 ml. was withdrawn from the flasks and run on to crushed ice in a 50 ml. polythene centrifuge tube which was immediately placed in the refrigerator at 0°. When all necessary samples had been taken, the tubes were removed from the refrigerator and exposed to room temperature until nearly all the ice had melted. The contents of each tube were then made to 50 ml. with ice-cold water and centrifuged at 0° for 10 min. at 11 000 g to separate the cells. Cells were resuspended in a measured volume of 0.03M phosphate buffer, pH 7.8.

Anaerobic incubations were carried out in Thunberg tubes under N_2 at 25°.

Growth experiments. In these experiments, which were designed to test the effect of different compounds on the growth of *Nitrobacter*, 25 ml. lots of medium G containing 70 μ g. of nitrite N/ml. as $NaNO_2$ (and, where appropriate, the compound under investigation) were sterilized in 100 ml. conical flasks at 120° for 20 min. and inoculated with a single loopful (approx. 0.01 ml.) of *Nitrobacter* culture. The flasks were then incubated at 28° and tested daily for the presence of nitrite. When the nitrite concentration of a culture had fallen below about 10 μ g. of nitrite N/ml., growth of *Nitrobacter* cells in the culture was presumed to have taken place and the time between inoculation and presumed growth was recorded.

Sodium cyanate was prepared from a commercial sample by repeated crystallization from boiling methanol–water (9:1, v/v).

Nitrourea was prepared by the method of Davis & Blanchard (1929).

Sodium chlorite was prepared according to Partington (1939). The purity of the final sample, estimated by the amount of I_2 liberated when the chlorite was added to a solution of excess KI, was about 85%.

All other reagents, with the exceptions of the dithionite and fluoracetate, were of AnalaR quality.

RESULTS

The oxygen uptake due to nitrite oxidation by *Nitrobacter* suspensions proceeded at a constant rate until almost all the nitrite had been oxidized; the rate then fell sharply almost to zero (Fig. 1). The oxygen uptake by *Nitrobacter* suspensions in the absence of nitrite was negligible (about 0.1 μ mole of O_2 /vessel/hr.). The rate of oxygen uptake varied with the initial nitrite concentration (Fig. 2). Estimations of nitrate carried out from time to time on the contents of experimental Warburg flasks showed that the amount of nitrite oxidized to nitrate always agreed, to within 5%, with the amount of oxygen consumed, according to the equation

Agreement between oxygen uptake and nitrite oxidation is therefore to be inferred even where it is not specifically mentioned.

Fig. 1. Oxygen uptake by *Nitrobacter* in the presence of nitrite. The Warburg vessel contained 2.5 ml. of *Nitrobacter* suspension in 0.03M phosphate buffer, pH 7.8. Sodium nitrite solution (0.5 ml.) was added from the side arm after temperature equilibration to give an initial NaNO_2 concentration of $4 \times 10^{-3}\text{M}$. The dotted horizontal line represents the O_2 uptake corresponding to complete oxidation of the added NO_2^- to NO_3^- .

Fig. 2. Effect of nitrite concentration on the rate of O_2 uptake by *Nitrobacter*. Experimental conditions were the same as those for Fig. 1 except that different amounts of NaNO_2 were added as 0.5 ml. of solution from the side arms to give the initial NaNO_2 concentrations shown on the abscissa. Oxygen uptakes were measured for each flask 30 min. after addition of the nitrite.

In the presence of chlorate, the rate of nitrite oxidation by *Nitrobacter* suspensions was not constant but gradually decreased as the experiment progressed (Fig. 3). In the experiments of Fig. 3, the chlorate was added to the *Nitrobacter* suspension in the main compartment of the Warburg vessels just before temperature equilibration; the nitrite was tipped in from the side arm about 10 min. later, i.e. just after temperature equilibration. Precisely similar results were, however, obtained either when the addition of nitrite from the side arm was delayed for 1 hr. after the chlorate and *Nitrobacter* had been mixed in the main vessel, or when the chlorate and nitrite were mixed in the side arm and added to the *Nitrobacter* together. Where this latter type of experimental procedure was used, the period for which the nitrite and chlorate remained mixed before addition to the *Nitrobacter* suspension did not appear to have any effect on the results.

Fig. 3. Effect of KClO_3 concentration on the rate of O_2 uptake by *Nitrobacter* in the presence of nitrite. Experimental conditions were similar to those for Fig. 1. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-3}M . The KClO_3 was added to the cell suspension in the main vessel before temperature equilibration; the concentrations of KClO_3 in the main vessels were: $1.7 \times 10^{-3}\text{M}$ (Δ); $8.3 \times 10^{-3}\text{M}$ ($\blacksquare$); $5.6 \times 10^{-3}\text{M}$ ($\bullet$); $4.2 \times 10^{-3}\text{M}$ ($\square$); nil ($\circ$).

The inhibition of nitrite oxidation caused by 8×10^{-3} M-KClO₃ remained virtually unchanged when the substrate concentration was raised from 2.5×10^{-3} M-NaNO₂ to 5×10^{-3} M-NaNO₂. Attempts to reverse the inhibition by nitrate were unsuccessful; concentrations of up to 5×10^{-2} M-NaNO₃ were tested. The effect of cell density on the inhibition of nitrite oxidation by chlorate is shown in Fig. 4.

When *Nitrobacter* suspensions were pre-incubated with chlorate alone (concentrations of up to 2×10^{-2} M-KClO₃ were tested) or with nitrite alone (up to 0.1 M-NaNO₂) for 1 hr., washed, and then tested in the Warburg apparatus, the ability of the suspensions to oxidize nitrite was unchanged. Cells pre-incubated with chlorate and nitrite together oxidized nitrite, after washing, at a constant rate. The rate lessened as the period of pre-incubation was increased (Table 1).

Fig. 4. Effect of cell density on the rate of O₂ uptake by *Nitrobacter* in the presence of nitrite and in the presence and absence of chlorate. Experimental conditions were similar to those for Fig. 1. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-2} M. Points ● and ■ indicate that 8×10^{-3} M-KClO₃ was present in main vessel, points ○ and □ indicate the absence of chlorate. The cell density of *Nitrobacter* used to determine points ○ and ● was three times that used to determine points □ and ■. The curves through points ● and ■ are not drawn as best fits to the points but are calculated from equation (c) (see Discussion). For the upper curve, $KQ_0 = 0.168$ μmole of O₂/min., for the lower curve, $KQ_0 = 0.062$ μmole of O₂/min.; these values are the slopes of the lines through points ○ and □. For both curves, $kZ = 0.019$; this value was obtained by inspection.

Fluoroacetate, nitrourea and various ions of general formula XO₃⁻ (other than ClO₃⁻ ions) were tested for their effect on nitrite oxidation by *Nitrobacter*. None proved as effective an inhibitor as chlorate, although the inhibition caused by nitrourea could be markedly increased, either by allowing freshly prepared nitrourea to stand in solution at room temperature for a period, or by treating the solution with alkali (Table 2). Chlorate,

Table 1. Effect of pre-incubation of *Nitrobacter* cells with nitrite and chlorate on nitrite-oxidizing activity

Cell suspensions were pre-incubated for various times with 10^{-2} M-NaNO₂ and 7×10^{-3} M-KClO₃ as shown, washed, and tested for nitrite-oxidizing activity under the experimental conditions of Fig. 1. Sodium nitrite was added from the side arm to give an initial concn. of 10^{-2} M.

Duration of pre-incubation (min.)	Rate of O ₂ uptake of the <i>Nitrobacter</i> suspension after pre-incubation (μmole of O ₂ /min.)
0 (control)	0.077
5	0.065
15	0.056
30	0.043
60	0.036
90	0.030

Table 2. Effect of various compounds on nitrite oxidation by *Nitrobacter*

Oxygen uptakes were measured under the experimental conditions of Fig. 3. A solution of the compound to be tested was added to the *Nitrobacter* suspension in the main vessel before temperature equilibration; no compound was added to the control vessels. Sodium nitrite was added from the side arm to give an initial concn. of 8×10^{-3} M in all vessels. Uptakes of oxygen were measured 1 hr. after addition of nitrite.

Compound tested	Concn. of compound (mM)	Percentage inhibition of O ₂ uptake
ClO ₃ ⁻	10	91
	2	80
BrO ₃ ⁻	10	15
	2	8
IO ₃ ⁻	10	6
	2	1
PO ₃ ⁻	10	2
	2	0
Fluoroacetate	10	32
	2	21
	1	16
Nitrourea, fresh	2	15
Nitrourea, stored 1 day at 15°	2	18
Nitrourea, stored 3 days at 15°	2	41
Nitrourea, alkali-treated*	2	68

* Treated with 10^{-2} M-NaOH for 1 hr. at 15°. Solution neutralized with HCl before testing.

bromate, iodate and fluoroacetate were also tested for their effect on freshly inoculated cultures of *Nitrobacter*. In these cultures, where the *Nitrobacter* population was small, all the compounds completely inhibited nitrite oxidation and therefore, presumably, growth, for a period of at least 9 days at concentrations of 4×10^{-4} M.

Cyanate proved to be a strong inhibitor of nitrite oxidation by *Nitrobacter*. Unlike the inhibition caused by chlorate, nitrite oxidation proceeded at a constant but diminished rate in the presence of cyanate. The degree of inhibition was directly related to the logarithm of the cyanate concentration (Fig. 5). The extent to which the nitrite-oxidizing activities of *Nitrobacter* suspensions were affected by pre-incubation with various mixtures of chlorate, cyanate and nitrite are shown in Table 3.

Chlorite proved to be an inhibitor of nitrite oxidation with an action somewhat between that of chlorate and that of cyanate; there was some increase in the degree of inhibition as the experiment progressed, but the increase was rather less obvious than it was with chlorate (Fig. 6).

Washed, aerated suspensions of *Nitrobacter* showed only very faint absorption bands when examined in a Hartridge reversion spectroscope. When nitrite was added to the suspensions to give a concentration of 10^{-2} M- NaNO_2 , however, three absorption bands immediately appeared. The sharpest and darkest was a band at $551 \text{ m}\mu$; this

was accompanied by a fainter, but almost equally sharp, band at $589 \text{ m}\mu$. and a diffuse band at $520\text{--}525 \text{ m}\mu$. Similar bands could be produced by the addition of dithionite instead of nitrite (Fig. 7). This fact, together with the wavelengths of the bands, has led us to refer to these bands as

Table 3. *Effect of pre-incubation of Nitrobacter cells with nitrite, cyanate, and chlorate, on nitrite-oxidizing activity*

Cell suspensions were pre-incubated for 30 min. with 10^{-2} M- NaNO_2 , 10^{-2} M- KClO_3 and 10^{-2} M- NaOCN where indicated (+), washed, and tested for nitrite-oxidizing activity under the experimental conditions of Fig. 1. Sodium nitrite was added from the side arm to give an initial concn. of 8×10^{-3} M.

Substances present during pre-incubation of the <i>Nitrobacter</i> suspension			Rate of O_2 uptake of the <i>Nitrobacter</i> suspension after pre-incubation ($\mu\text{mole of O}_2/\text{min.}$)
NaOCN	NaNO_2	KClO_3	
-	-	-	0.29
-	+	-	0.32
+	-	-	0.30
-	-	+	0.29
+	+	-	0.31
-	+	+	0.17
+	-	+	0.30
+	+	+	0.28

Fig. 5. Effect of cyanate on nitrite oxidation by *Nitrobacter*. Experimental conditions were similar to those for Fig. 1. Sodium cyanate was added at various concentrations to the *Nitrobacter* suspension in the main vessel. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-2} M. In the presence and absence of NaOCN , O_2 uptake was linear with time.

Fig. 6. Effect of KClO_2 concentration on the rate of O_2 uptake by *Nitrobacter* in the presence of nitrite. Experimental conditions were similar to those for Fig. 1. Sodium nitrite was added from the side arm to give an initial concentration of 10^{-2} M. The concentrations of KClO_2 in the main vessels were: 3×10^{-3} M ($\blacksquare$); 10^{-3} M ($\bullet$); 3×10^{-4} M ($\square$); nil ($\circ$).

'cytochrome bands'. Suspensions of the heterotrophic contaminant of the *Nitrobacter* cultures (see Materials and Methods) showed no such bands on treatment with nitrite. *Nitrobacter* suspensions treated with 10^{-2} M-KClO₃, or with 10^{-2} M-NaOCN followed by 10^{-2} M-NaNO₂, showed no cytochrome bands, but the bands could nevertheless be generated by nitrite or dithionite after such suspensions had been washed with water at the centrifuge. *Nitrobacter* suspensions treated with 10^{-2} M-NaNO₂ plus 10^{-2} M-KClO₃ immediately showed the cytochrome bands, but the bands (particularly that at 551 m μ .) gradually faded if the cells were allowed to remain in contact with the nitrite and chlorate. *Nitrobacter* suspensions whose bands had been allowed to fade in this way failed, after water washing and treatment with nitrite or dithionite, to show cytochrome bands of intensity equal to those shown by a similar suspension not incubated with chlorate and nitrite. The diminution of intensity of the 551 m μ . band, brought about by incubation of the cells with chlorate and nitrite, ran parallel with diminution of nitrite-oxidizing activity (Fig. 8). Chlorite added to a *Nitrobacter* suspension produced an immediate disappearance of the cytochrome bands; the bands could not be regenerated in cells treated with chlorite by water washing at the centrifuge followed by treatment with nitrite or dithionite.

In attempts to find some electron acceptor (other than atmospheric oxygen) for nitrite oxidation by *Nitrobacter*, cell suspensions, together with nitrite in 0.03 M phosphate buffer, pH 7.8, were incubated anaerobically for 1 hr. in the presence of triphenyl-tetrazolium chloride, methylene blue, methylene

Fig. 7. Absorption spectrum of *Nitrobacter* cells in the presence of dithionite. Optical densities recorded are difference values recorded against the 'blank' values given by a thoroughly aerated *Nitrobacter* suspension containing no dithionite. Similar spectra were given by *Nitrobacter* suspensions made 10^{-2} M with respect to NaNO₂.

Fig. 8. Relation between cytochrome 551 content and nitrite-oxidizing ability of *Nitrobacter*. The cytochrome 551 initially present in the *Nitrobacter* cells was partially destroyed by pre-incubation of the cells with 10^{-2} M-KClO₃ and 10^{-2} M-NaNO₂. The amount of cytochrome 551 remaining in the cells after pre-incubation was determined as the ratio 'optical density at 551 m μ : optical density at 589 m μ .' (see Materials and Methods) after treatment with dithionite. The nitrite-oxidizing ability of the pre-incubated cells was determined under experimental conditions similar to those for Fig. 1.

Fig. 9. Relation between nitrite disappearance from, and chlorate concentration of, anaerobic suspensions of *Nitrobacter*. Cells were incubated for 1 hr. at 25° in Thunberg tubes in the presence of 2×10^{-3} M-NaNO₂ and various concentrations of KClO₃, under an atmosphere of N₂.

blue plus adenosine triphosphate, mammalian cytochrome *c*, water extract of baker's yeast, or chlorate. The nitrite concentration remained unchanged in all preparations except that containing chlorate; here there was a slight fall in nitrite concentration. This fall did not occur when boiled *Nitrobacter* suspensions were used. Further experiments showed that the fall in nitrite concentration was related to the concentration of chlorate used (Fig. 9).

DISCUSSION

The work here described is, to the best of our knowledge, the first detailed biochemical investigation of the mechanism whereby *Nitrobacter* oxidizes nitrite to nitrate. Because there is as yet no method of growing *Nitrobacter* in reasonable quantity, our results are to some extent fragmentary. Nevertheless, in view of our findings we feel justified in suggesting that the following reactions occur during nitrite oxidation by *Nitrobacter*:

- *(i) $\text{Fe}_{\text{cyt. 551}}^{3+} + \text{NO}_2^- \rightarrow \text{Fe}_{\text{cyt. 551}}^{2+} + \text{NO}_2$.
- (ii) $\text{Fe}_{\text{cyt. 551}}^{2+} + \text{NO}_2 + \frac{1}{2}\text{O}_2 \rightarrow \text{Fe}_{\text{cyt. 551}}^{3+} + \text{NO}_3^-$.
- (iii) $\text{Fe}_{\text{cyt. 551}}^{2+} + \text{NO}_2 + \text{ClO}_3^- \rightarrow \text{Fe}_{\text{cyt. 551}}^{3+} + \text{NO}_3^- + \text{ClO}_2^-$.
- (iv) $\text{Fe}_{\text{cyt. 551}}^{3+} + \text{ClO}_2^- \rightarrow \text{inactivated cytochrome 551}$.

* Reaction (i) is reversibly inhibited by cyanate.

This scheme is intended as an outline, not as a precise formulation, of the processes taking place in *Nitrobacter*. The symbol NO_2 does not necessarily represent the nitrite radical, it merely represents some compound, free or bound to a carrier, at the oxidation level of the nitrite radical. Although the reactions are depicted as taking place directly, this does not preclude the possibility that one or more of them may involve carrier systems. Our evidence for the scheme is as follows:

Reaction (i). $\text{Fe}_{\text{cyt. 551}}^{2+}$ is produced when nitrite is added to *Nitrobacter* suspensions and the rate of nitrite oxidation depends on the amount of cytochrome 551 in the cell (Fig. 8). Addition of cyanate to *Nitrobacter* suspensions containing nitrite inhibits nitrite oxidation (Fig. 5) and, if present in sufficiently high concentration (see Results), prevents the appearance of the band of $\text{Fe}_{\text{cyt. 551}}^{2+}$. The effect of cyanate is, however, reversible by water washing. These results suggest that cyanate has no effect on the cytochrome 551 itself, but prevents the cytochrome 551 from accepting electrons from nitrite.

Reaction (ii). This reaction (i.e. the reoxidation of $\text{Fe}_{\text{cyt. 551}}^{2+}$ by atmospheric oxygen) must take place, although it may not take place directly; it may involve intermediate carriers. In the absence of

nitrite, the spectrum of a *Nitrobacter* suspension shows no reduced cytochrome bands; these bands appear immediately when nitrite is added to the suspension and disappear when all the nitrite has been oxidized. This suggests that reaction (ii) rather than reaction (i) limits the rate of nitrite oxidation in *Nitrobacter*. That reaction (ii) is slower than reaction (i) also harmonizes with the fact that, although the nitrite concentration in a *Nitrobacter* suspension steadily falls as nitrite is oxidized to nitrate, the rate of nitrite oxidation by such suspension remains fairly constant until almost all the nitrite has been oxidized (Fig. 1).

Reaction (iii). That chlorate can act as an oxidizing agent in nitrite oxidation by *Nitrobacter* follows from the results of Fig. 9, which show that nitrite disappears from *Nitrobacter* suspensions under anaerobic conditions in the presence of chlorate. Owing to the fact that nitrate cannot be estimated satisfactorily in the presence of relatively large amounts of chlorate, we were not able to show that the nitrite disappearing was oxidized to nitrate, but since chlorate cannot easily be visualized as a reducing agent, it seems almost certain that the nitrite was indeed so oxidized. Indirect evidence for the occurrence of reaction (iii) is provided by the fact that chlorate inhibition of nitrite oxidation only becomes apparent when nitrite oxidation is actually proceeding, during which process the inhibition becomes progressively greater (Table 1; Figs. 3, 4).

Reaction (iv). The rapid destruction of cytochrome 551 by chlorite has been observed spectroscopically; it seems probable that this destruction is similar to the destruction of metmyoglobin observed by George & Irvine (1954). Presumably because it destroys cytochrome 551, chlorite proved to be a powerful inhibitor of nitrite oxidation by *Nitrobacter* (Fig. 6).

Since all our results accord with the operation of reactions (i)–(iv), it seems probable that these reactions describe reasonably well the mechanism of nitrite oxidation in *Nitrobacter*. There are, however, some ancillary points worth consideration.

Simpson (1955) has shown that the kinetics of the inhibition of nitrite oxidation by chlorate may be adequately explained on the basis of two postulates:

(a) The rate of nitrite oxidation in a *Nitrobacter* suspension is proportional to the amount (Q_t) of cytochrome 551 in the cells at time ' t ' (see Fig. 8), i.e.

$$-\frac{d(\text{NO}_2)}{dt} = KQ_t,$$

where K is a constant and (NO_2) is the amount of nitrite.

(b) The rate of destruction of cytochrome 551 in a *Nitrobacter* suspension containing chlorate at

concentration Z is proportional to Z and to Q_t (see reactions (iii) and (iv)), i.e.

$$-\frac{dQ}{dt} = kZQ_t,$$

where k is a constant. Integration and combination of these two expressions yields the equation:

$$(c) \quad -(\text{NO}_2)_t = \frac{KQ_0}{kZ} (1 - e^{-kZt}),$$

where $-(\text{NO}_2)_t$ = amount of nitrite oxidized after time ' t '; KQ_0 = initial rate of nitrite oxidation, i.e. = rate of nitrite oxidation in the absence of chlorate. The fit of equation (c) to the experimental points is shown in Fig. 4. When ' t ' is very large, equation (c) approximates to

$$(d) \quad -(\text{NO}_2)_t = \frac{KQ_0}{kZ}.$$

This equation (d) predicts that, for a given concentration of chlorate, the amount of nitrite oxidized to nitrate before oxidation becomes vanishingly slow depends on the value of Q_0 ; that is, it depends on the cell density of the *Nitrobacter* suspension used. This prediction not only agrees with the results of Fig. 4, it also offers a possible explanation of the results of Lees & Quastel (1945), which showed that chlorate at concentrations of about 10^{-5}M virtually prevented nitrite oxidation in soils with a low *Nitrobacter* population, but had little effect on nitrite oxidation in soils with a high *Nitrobacter* population. Lees & Quastel also found that the inhibitory action of 10^{-5}M chlorate could be abolished by nitrate at a concentration of about 10^{-2}M . These results are explicable on the basis of reaction (iii). Since nitrate is an end product of the reaction, the forward reaction should be inhibited by nitrate. Thus in the presence of nitrate, chlorite formation should be minimized and destruction of cytochrome 551 should be reduced. Our failure to diminish the inhibitory effect of chlorate by addition of nitrate may be explained by the fact that, at the high cell densities of *Nitrobacter* used in the present experiments, no possible concentration of nitrate could appreciably reverse reaction (iii). Lees & Quastel (1945) reversed chlorate inhibition of nitrite oxidation by a molar ratio of chlorate: nitrate of approximately 1: 10^3 . Since the chlorate concentrations in our experiments have been of the order of 10^{-2}M , no reversal by nitrate could have been expected much below a concentration of 10M nitrate. At this concentration of nitrate *Nitrobacter* could scarcely be expected to operate at all. Considerations of cell density probably explain also why iodate and bromate, although they only weakly inhibited nitrite oxidation in experimental *Nitrobacter* suspensions (Table 2), strongly inhibited oxidation in freshly inoculated cultures (see

Results). Quastel & Scholefield (1951) found that even in soils with a high *Nitrobacter* population, potassium chlorate at a concentration of $6 \times 10^{-3}\text{M}$ inhibited nitrite oxidation. This agrees with the results presented here. The failure of fluoroacetate to inhibit nitrite oxidation by *Nitrobacter* (Table 2), although it inhibited growth of *Nitrobacter*, is probably to be explained on the grounds that it interfered with the mechanisms of the organisms which assimilate carbon dioxide rather than with their nitrite-oxidizing mechanisms.

Nitrourea in aqueous solution decomposes to cyanate, especially under alkaline conditions (Davis & Blanchard, 1929). This probably explains why solutions of nitrourea inhibited nitrite oxidation more strongly after standing or alkali treatment (Table 2). The reported inhibition by nitrourea of nitrite oxidation by *Nitrobacter* in soil (Quastel & Scholefield, 1951) may likewise be due to the transformation of nitrourea into cyanate during percolation through the soil.

We are now trying to grow *Nitrobacter* on a scale sufficiently large to enable us to separate the cytochrome systems. By this means we hope to study more closely the details of the mechanisms outlined in reactions (i)–(iv).

SUMMARY

1. The mechanism of nitrite oxidation has been studied in the autotrophic bacterium *Nitrobacter*.
2. Cyanate inhibited nitrite oxidation in *Nitrobacter*; the effect was reversible by washing the cells with water.
3. Chlorate did not inhibit nitrite oxidation directly but was converted, during the course of nitrite oxidation, into some compound (possibly chlorite) that did inhibit nitrite oxidation. This inhibition could not be reversed by washing with water.
4. The results of these studies with cyanate and chlorate, together with results from spectroscopic examination of *Nitrobacter* cells under various conditions, suggest that a cytochrome with an absorption maximum at $551\text{ m}\mu$. in the reduced state is intimately concerned with nitrite oxidation by *Nitrobacter*.

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The Annelid Phosphokinases

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Roche and his co-workers (Thoai, Roche, Robin & Thiem, 1953; Thoai & Robin, 1954) have described the isolation of taurocyamine and glycocyamine from a number of annelid worms and this work has been confirmed by Ackermann (1955*a, b*). It was suggested that these bases when phosphorylated on the terminal amino group functioned as 'phosphagens'. Thoai *et al.* (1953) and Hobson & Rees (1955) showed that these bases and creatine were present as the phosphates in annelid muscle. Further support for this concept is given by the evidence presented in this paper, that the annelids contain phosphokinases capable of phosphorylating taurocyamine, glycocyamine and creatine at the expense of adenosine triphosphate (ATP); the reactions can be expressed as a generalized version of the Lohmann reaction: guanidine base + ATP $\rightleftharpoons$ phosphagen + ADP. A preliminary report of this work was presented by Hobson (1955).

METHODS

Extraction of enzymes. The animal was cooled in sea water until it was torpid; the gut was removed where this was practicable. To the weighed muscle of the body wall was added 2 vol. of ice-cold distilled water and the tissue was ground in a cold mortar with a little acid-washed sand. The extract was allowed to stand at 0° for 10 min. and then 5 vol. of cold acetone was added with stirring. The extract was filtered and the acetone-dried powder placed in a vacuum desiccator over anhydrous CaCl₂. The powder proved stable for several months if stored at 2°.

For the transphosphorylation experiments 2 g. of the acetone powder was ground with 10 ml. of distilled water and the extract dialysed against water for 15 hr. at 0°. The insoluble material was removed by centrifuging and the clear supernatant used for the experiments.

Transphosphorylation system. This was based on the system described by Lardy, Kuby & Noda (1954*a, b*) for

their studies on creatine phosphokinase. The final concentrations present were: guanidine base, 0.024 M; ATP, 0.005 M; MgSO₄, 0.0003 M; glycine buffer, 0.1 M (pH 9.0); 0.4 ml. of enzyme soln. was added. The final vol. was 3 ml. and incubation was for 15 min. at 40°. This system was found to give maximum phosphorylation, the addition of all components being necessary for phosphorylation of the base. The reaction was completed in about 10 min. and there was no evidence of inactivation of the enzyme at this temperature nor of any breakdown of the phosphagens before analysis.

Materials. Creatine, taurocyamine and glycocyamine were commercial preparations (L. Light and Co. Ltd.). ATP was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). Creatine phosphate was prepared by the method of Ennor & Stocken (1948).

Determinations. The quantity of phosphagens was determined after transphosphorylation experiments as follows. The reaction was stopped by the addition of 2 ml. of ice-cold 20% (w/v) trichloroacetic acid to the incubation mixture. The mixture was cooled for 5 min. in crushed ice and then centrifuged at 600 g for 5 min. at 0°. The clear supernatant was neutralized (pink to phenolphthalein) and the Ca(OH)₂-CaCl₂ reagent of Fiske & Subbarow (1929) was added to precipitate the inorganic and nucleotide phosphates. After standing for 10 min. in ice the tubes were centrifuged. The phosphagens were estimated in the supernatants by the method described by Baldwin & Yudkin (1950).

The solvents used in chromatographic analysis of the guanidine phosphates and guanidine bases were as described by Hobson & Rees (1955). The chromatograms were run at 26.5° and not, as reported earlier by us, at 22°.

RESULTS

Enzyme extracts were prepared from a number of errant and sedentary polychaete worms and portions (0.4 ml.) were incubated in the standard medium with each of the four substrates, taurocyamine, glycocyamine, arginine and creatine. The