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*WATER AS THE SOURCE OF OXIDANT AND REDUCTANT IN BACTERIAL CHEMOSYNTHESIS**

BY M. I. H. ALEEM, G. E. HOCH, AND J. E. VARNER

RESEARCH INSTITUTE FOR ADVANCED STUDIES, BALTIMORE, MARYLAND

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The two vital aspects of chemoautotrophic metabolism are the generation of energy (ATP or the equivalent) and a simultaneous production of reducing power coupled to the enzymic oxidation of an inorganic substrate. Aleem and Nason[1] reported that in the obligately chemoautotrophic bacterial genus *Nitrobacter* the enzymic oxidation of nitrite is catalyzed by a cytochrome-containing electron transport particle via cytochrome *c* and cytochrome oxidase-like components. They subsequently demonstrated[2] the coupling of this oxidation with the generation of high-energy phosphate bonds which were identified as adenosine triphosphate (ATP). The over-all reaction is:

$$NO_2^- + {}^1/_2\,O_2 + nADP + nP_i \xrightarrow[\text{transport chain}]{\text{cytochrome electron}} NO_3^- + nATP. \quad (1)$$

The energy liberated in the above equation is utilized in part to drive the endergonic reduction and assimilation of carbon dioxide,[3] and in part to produce reduced pyridine nucleotides which are essential for the operation of the carbon reduction cycle.[3, 4] The following equations represent these events:

$$3ATP + PN^+ + H^+ + 2\ \text{ferrocytochrome}\ c \rightarrow 3ADP + 3P_i + PNH + 2\ \text{ferricytochrome}\ c \quad (2)$$

$$CO_2 + 3ATP + 2H^+ + 2PNH \rightarrow [CH_2O] + H_2O + 2PN^+ + 3\,ADP + 3P_i. \quad (3)$$

The process of chemosynthesis as displayed by *Nitrobacter*, therefore, appears to be analogous to photosynthesis. In this system as in photosynthesis, water must be the source of hydrogen for the pyridine nucleotide reduction.

The experiments reported below demonstrate for the first time that water (not molecular oxygen) participates in the oxidation of nitrite to nitrate and that the hydrogen donor for the concomitant reduction of pyridine nucleotide is also water. The over-all stoichiometry of the reaction is represented by the following equation:

$$NO_2^- + H_2O^{18} + A \rightleftarrows NO_3^{18-} + AH_2. \quad (4)$$

Materials and Methods.—Cells of *Nitrobacter agilis* (American Type Culture Collection no. 9482) were grown and harvested as described by Aleem and Alexander.[5] Nitrite oxidation was estimated by its disappearance[1] as well as by measuring the nitrate formed using a modified disulfonic acid method.[6]

For the measurement of O^{18} incorporation into nitrate, *Nitrobacter* cells were incubated with H_2O^{18} or O_2^{18} in the presence of nitrite. At the completion of the reaction, the cells were centrifuged out and washed once with 5 ml of distilled water. The resulting supernatant fractions were mixed, lyophilized, and taken up in 10 ml of distilled water. The solution was run through Dowex 50 (H^+-form) with 10 ml bed volume, and the effluent was titrated to pH 7.0 with 1 *N* NH_4OH. Then 1000 μmoles (80 mg) of NH_4NO_3 was added as carrier, and the solution was lyophilized. The NH_4NO_3 residue plus some NH_4Cl from the residual Tris-HCl buffer (see the reaction mixture) was heated in a sealed Pyrex tube for 3 hr at 300°C in order to obtain H_2O^{18} as described by Friedman and Bigeleisen:[7]

$$NH_4NO_3^{18} \rightarrow N_2O^{18} + 2\,H_2O^{18}.$$

A 35-μl volume of this H_2O^{18} was electrolyzed after the addition of 65 μl of 1 *N* KOH in a special inlet vessel on the mass spectrometer.[8] The atom per cent O^{18} was obtained as the ratio of mass 34 to mass 34 + mass 32.

Exchange reactions between nitrite and water, and nitrate and water were measured after stopping the reaction with 0.1 ml of 10 *M* KOH. The water was collected by vacuum distillation and assayed for O^{18} as described above.

Ferricyanide reduction was measured spectrophotometrically by the decrease in absorbance at 420 mμ. The NADH formed during the oxidation of nitrite was trapped as radioactive lactate produced by using a pyruvate-lactic dehydrogenase trapping system in the presence of tritiated water and 50 mg carrier lactate.[3]

Results.—The source of oxygen for nitrite oxidation: It might be considered that molecular oxygen should oxidize nitrite during chemosynthesis by *Nitrobacter*. However, the data of Table 1 show that when nitrite is oxidized in the presence of O_2^{18}, there is only a small recovery of O^{18} in the nitrate. This amount is far below

TABLE 1

OXIDATION OF NITRITE BY INTACT *Nitrobacter* CELLS IN THE PRESENCE OF OXYGEN-18

Treatment	O^{18} atom % in NO_3^-	O^{18} Atom % Excess Observed	O^{18} Atom % Excess Theoretical
Expt. I			
Cells + NO_2^- + air	0.206 ± 0.002	—	—
Cells + NO_2^- + 30% O_2^{18} + 70% N_2	0.216 ± 0.002	0.010	4.30
Expt. II			
Cells + NO_2^- + air	0.206 ± 0.001	—	—
Cells + NO_2^- + 30% O_2^{18} + 70% N_2	0.212 ± 0.002	0.006	4.30

Reaction mixture in a total volume of 50 ml contained 10 ml of *Nitrobacter* cell suspension (containing 5 mg protein/ml), 5 mmoles of tris(hydroxymethyl)aminomethane buffer, pH 8.0, 1000 μmoles nitrite, 30% by volume of 24 atom % excess O^{18}-O_2, and the remaining gas phase was oxygen-free N_2. Reaction flask was shaken at 30°C for 60 min; all the nitrite was converted to nitrate during this period. The $NH_4NO_3^{18}$ was recovered, using techniques described under *Materials and Methods*. No carrier was added.

that expected from incorporation of an oxygen atom into nitrite. The incorporation observed could be due to the reduction of O_2^{18} to H_2O^{18} followed by incorporation of this greatly diluted label.

The alternative is that the oxygen for nitrite oxidation comes from water. The results in Table 2 clearly show that the oxygen from H_2O^{18} appeared in the nitrate

TABLE 2

OXIDATION OF NITRITE BY INTACT *Nitrobacter* CELLS IN THE PRESENCE OF H_2O^{18}

Treatment	O^{18} atom % in NO_3^-	O^{18} Atom % Excess Observed	O^{18} Atom % Excess Theoretical
Expt. I			
Cells + NO_2^-	0.206 ± 0.002	—	—
Cells + NO_2^- + H_2O^{18}	0.250 ± 0.002	0.044	0.032
Expt. II			
Cells + NO_2^-	0.206 ± 0.002	—	—
Cells + NO_2^- + H_2O^{18}	0.284 ± 0.002	0.078	0.064

Reaction mixture in a total volume of 5.0 ml contained 4.5 ml of *Nitrobacter* cell suspension (containing 5 mg protein/ml), 200 μmoles nitrite, 400 μmoles tris(hydroxymethyl)aminomethane buffer pH 8.0. Expts. I and II received, respectively, 0.1 and 0.2 ml H_2O^{18}, 82 atom % excess O^{18}. Reaction vessel was shaken in the presence of air at 30°C for 30 min; all the added nitrite was converted to nitrate during this period. Other conditions were the same as described in Table 1, except that 80 mg NH_4NO_3 was added as carrier (see *Materials and Methods*).

formed from nitrite oxidation. The observed values of atom % excess O^{18} found in nitrate-oxygen are in slight excess of the theoretical values. This probably arises from a limited exchange of O^{18} between nitrite and H_2O as shown in the following data.

Exchange between oxygens of nitrate, nitrite, and water: The foregoing results and discussion suggest that the O^{18} appearing in nitrate from H_2O^{18} might arise from the oxygen exchange reactions between water and nitrate. The results in Table 3 show

TABLE 3

O^{18}-EXCHANGE BETWEEN NO_3^{18-}, NO_2^{18-}, AND H_2O

Treatments	O^{18} atom % in H_2O	O^{18} atom % excess*
Na_2SO_4 standard	0.205 ± 0.002	—
Cells + NO_3^{18-}	0.207 ± 0.002	0.002
Cells + NO_3^{18-} (anaerobic)	0.205 ± 0.002	0.000
Cells + NO_2^{18-}	0.213 ± 0.002	0.008
Cells + NO_2^{18-} (anaerobic)	0.214 ± 0.002	0.009
NO_2^{18-} — cells (control)	0.205 ± 0.002	—

Reaction mixture in a total volume of 1.0 ml contained 0.02 *M* KNO_3^{18} (84 atom % O^{18} excess) or 0.02 *M* KNO_2^{18} (80 atom % O^{18} excess), 0.1 ml cells containing about 2 mg protein, and 50 μmoles Tris-HCl buffer, pH 8.0. Reaction mixture was incubated for 90 min at 30°C. Reaction was stopped by adding 0.1 ml of 10 *M* KOH.

* A complete exchange of O^{18} from KNO_3^{18} or KNO_2^{18} would correspond to 0.091 or 0.058 atom % O^{18} excess, respectively.

TABLE 4

NITRITE OXIDATION BY *Nitrobacter* INTACT CELLS AND CELL-FREE EXTRACTS IN THE PRESENCE OF OXYGEN OR FERRICYANIDE AS TERMINAL ELECTRON ACCEPTORS

Treatment	Nitrite Oxidized (μmoles) Terminal Electron Acceptor: Ferricyanide	Oxygen	Ferricyanide reduced (μmoles)
Cell-free extract	2.62	3.92	4.4
Intact cells	4.20	5.00	7.2

Reaction mixture in a total volume of 2.0 ml contained 0.1 ml cells (10 mg protein) or 0.5 ml of the supernatant from a cell-free extract centrifuged at 20,000 × *g* for 30 min (5 mg protein), 5.0 μmoles KNO_2, 10 μmoles potassium ferricyanide, and 150 μmoles Tris-HCl buffer, pH 8.0. No ferricyanide was used in the presence of oxygen. In the presence of ferricyanide the reaction vessel was evacuated and gassed with oxygen-free N_2 (repeated three times) and nitrite was then tipped in to start the reaction. The reaction was stopped after incubation for 30 min at 30°C.

that this was not the case. No significant oxygen exchange between water and nitrate was catalyzed under aerobic or anaerobic conditions by *Nitrobacter* cells.

Table 3 also shows that some exchange (but not complete exchange) occurred between nitrite and water under aerobic and under anaerobic conditions; this probably accounts for the slight excess of O^{18} incorporation reported in Table 2.

Anaerobic nitrite oxidation in the presence of ferricyanide: A different approach was used to show that nitrite oxidation by *Nitrobacter* intact cells or cell-free extracts could proceed in the absence of molecular oxygen, provided the latter were replaced by an artifical electron acceptor such as ferricyanide. The data in Table 4 indicate such an oxidation occurs with ferricyanide as the terminal electron acceptor. The stoichiometry of nitrite oxidized and ferricyanide reduced was reasonable.

Discussion.—There have been speculations concerning the source of reducing power in chemosynthetic bacteria.[9, 10] Ultimately, water must be the source of reducing power in the *Nitrobacter* system. The NADH generated in the presence of tritiated water when trapped by pyruvate and lactic dehydrogenase (LDH) yields labeled lactate.[3] Much more tritium was incorporated into added unlabeled lactate formed in the presence of added NAD^+, pyruvate-LDH trapping system, and nitrite than into added lactate in the absence of nitrite. These results[3] clearly show that in the *Nitrobacter* system, water not only serves as a reductant for pyridine nucleotide but also as an oxidant for nitrate.

The following reactions have been reported in a chemosynthetic bacterium:

$$NO_2^- + nADP + nP_i + {}^1/_2\,O_2 \xrightarrow[\text{transport chain}]{\text{cytochrome electron}} NO_3^- + nATP \qquad (1)$$

$$NAD^+ + 2H^+ + 2e^- + \text{energy} \rightarrow NADH + H^+ \qquad (2)$$

$$NADH + H^+ + ADP + P_i + {}^1/_2\,O_2 \rightarrow NAD^+ + ATP + H_2O. \qquad (3)$$

In reaction (1) the nitrite donates electrons to the cytochrome-electron transport chain. These electrons are ultimately accepted by molecular oxygen with concomitant phosphorylation (Aleem and Nason).[1, 2] The energy generated in reaction (1) is used to drive a portion of the electrons donated by nitrite to the level of pyridine nucleotide. The energy-dependent reduction of pyridine nucleotide in reaction (2) involves the participation of electrons donated by a reduced component (i.e., ferrocytochrome *c*) of the respiratory chain, and protons donated by water (Aleem *et al.*[3, 4]). The requirements for the energy utilized in reaction (2) could be fulfilled by reaction (3).

In this report we have shown that nitrite can be oxidized via a dehydrogenation. It seems likely that the nitrite molecule (or some activated form of it) is hydrated prior to electron removal.

Summary.—In the chemoautotroph *Nitrobacter agilis*, the oxygen of water is responsible for the oxidation of nitrite. This process is independent of molecular oxygen. These results constitute the first experimental demonstration that water serves as the source of oxygen in chemoautotrophic bacteria.

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ALTERATIONS IN POLYRIBOSOMES OF RETICULOCYTES MATURING IN VIVO

BY D. DANON, T. ZEHAVI-WILLNER,* AND G. R. BERMAN

SECTION OF BIOLOGICAL ULTRASTRUCTURE, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL

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The alterations that occur in reticulocytes in the process of maturation into erythrocytes, during which their capacity to synthesize hemoglobin is progressively lost, have been studied using various approaches. Early studies described the progressive disappearance of the reticulum while the size of the cells is reduced and their osmotic fragility increased. The "substantia reticulo filamentosa" was later found to be rich in RNA and the reduction in RNA content of the cell was associated with its decline in capacity to synthesize hemoglobin.[1] Since membrane-bound[2–4] as well as free[5–7] ribosomes have been established as the site of protein synthesis and polyribosomes have been recognized as the site of protein synthesis in reticulocytes,[8–12] several studies have been undertaken in order to elucidate the fate of the ribosomes in the process of maturation.

Loss of cytoplasmic ribonucleoprotein has been noted during maturation in experiments with various cells.[13] Marks *et al.*[14] reported that during *in vitro* maturation of reticulocytes, a decrease in polyribosome content was found to be associated with the loss of the cells' capacity to synthesize proteins. Rifkind *et al.*[15] analyzed the alterations in polyribosome content and structure which occur as erythroid cells mature *in vitro* as well as in Millipore chambers *in vivo*. They showed that as maturation