PRODUCTION AND UTILIZATION OF NITROUS OXIDE BY *PSEUDOMONAS DENITRIFICANS*

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Nitrous oxide has been recognized as a product of denitrifying organisms since the early work of Gayon and Dupetit (1882*a, b*; 1886). Under some conditions it can be the major initial product (Wijler and Delwiche, 1954). Kluyver and Verhoeven (1954) have investigated the production of nitrous oxide by a large number of denitrifiers and conclude that its production and utilization is characteristic of all denitrifiers.

Sacks and Barker (1952) observed that under some conditions the utilization of nitrous oxide by nitrate adapted cells of *Pseudomonas denitrificans* showed a lag, indicating that adaptation to nitrate did not necessarily include adaptation to nitrous oxide. They further observed that azide and dinitrophenol inhibited the reduction of nitrous oxide but did not affect the reduction of nitrate to nitrogen gas. On the basis of this observation they concluded that, at least for this organism, nitrous oxide was not an obligatory intermediate. Allen and van Niel (1952) working with *Pseudomonas stutzeri* did observe simultaneous adaptation of this organism to nitrate and nitrous oxide. They found also that under conditions of cyanide inhibition the reduction of nitrate and nitrous oxide could be suppressed completely but the organism would still produce nitrogen from nitrite. On the basis of these findings, the conclusion is implied that nitrous oxide is not an intermediate in the production of nitrogen gas from nitrite but that it is reversibly derived from some intermediate product in the denitrification reaction.

Kluyver and Verhoeven (1954) conclude that nitrous oxide normally is intermediate in the denitrification process because of the ubiquity of its occurrence, but suggest that there are two possible paths of denitrification and that an alternative direct hydrogenation of an intermediate having the empirical formula $N_2O_2H_2$ would explain the results of Sacks and Barker (1952), and Allen and van Niel (1952).

These studies of the denitrification reaction have been by manometric techniques and dependent upon the differential solubilities of nitrogen and nitrous oxide. For the experiments to be described, the exchange of gases by denitrifying organisms has been followed, using the mass spectrometer to identify gases in the reaction. The production and utilization of nitrous oxide and the formation of nitrogen have been followed with resting cell suspensions from cultures grown under various levels of nitrate, nitrite, oxygen, or nitrous oxide. In this manner it has been possible to gather further information relative to adaptation phenomena and the effect of inhibitors in this reaction.

EXPERIMENTAL METHODS

The organism used was a strain of *Pseudomonas denitrificans* obtained from Dr. L. E. Sacks. Cells were grown for 24 hr in 1-L Florence flasks containing 200 ml of a medium consisting of 0.6 per cent glucose, 0.5 per cent yeast extract, 0.5 per cent phosphate buffer, pH 6.8, and 0.1 volume per cent of a mixture of trace elements including iron and molybdenum dissolved in 0.25 per cent ethylenediaminetetraacetic acid. Nitrate, nitrite, nitrous oxide, or oxygen were provided as hydrogen acceptors, depending upon the experiment and as described below. Gases when used were bubbled through the medium at the rate of approximately 10 ml per min.

Cells were incubated at 35 C, harvested by centrifugation, washed once with 0.1 per cent potassium chloride, and suspended in 9 times their wet weight of 0.1 per cent potassium chloride. One ml of this cell suspension was then placed in a 25-ml Thunberg tube adapted to have an additional side arm in order that substrate could be added after the tubes were evacuated. A folded piece of filter paper, moistened with 5 per cent sodium carbonate, was placed in the tube top to absorb respired carbon dioxide. Tubes were filled with an appropriate atmosphere of nitrous oxide and argon at a pressure of 200 mm of

mercury, and substrate and cell suspension mixed. The argon served as a reference gas so that the disappearance of nitrous oxide could be followed by observing the change in 44:40 ratio mass spectrometrically. The formation of nitrogen was observed by measuring the 28:40 ratio in a sample from which nitrous oxide was first removed by freezing in liquid nitrogen. In those cases where N^{15}-labeled nitrate was included in the substrate, the conversion of nitrate to nitrous oxide or nitrogen was followed by recording the 45:44 and 29:28 ratios, respectively.

RESULTS

An initial experiment was conducted to determine the comparative effects of different growth conditions on the ability of cells to utilize nitrous oxide. *P. denitrificars* cultures were grown, one flask using nitrate as a hydrogen acceptor, at a concentration of 0.05 M, and one flask nitrous oxide. Comparable yields were obtained under both sets of conditions, although generally nitrous oxide produced the most luxuriant growth. This is probably explained by the high solubility of nitrous oxide and toxic effects sometimes resulting from higher concentrations of nitrate. The shift in pH of the medium as nitrate ion is destroyed may also affect growth adversely. Cells obtained in this manner were separated by centrifugation, washed, and suspended. To tubes containing 1 ml of a 10 per cent cell suspension and 1 ml of nitrate-free medium were added 10 μmoles of N^{15}-labeled nitrate and an atmosphere of 175 μmoles of nitrous oxide. Disappearance of nitrous oxide and formation of nitrogen are shown in figure 1A. Nitrous oxide grown cells immediately and rapidly utilized nitrous oxide and released nitrogen therefrom, whereas nitrate grown cells showed some lag. N^{15} from nitrate appeared in the nitrous oxide and nitrogen as shown in figure 1B. Here a quantitative interpretation of the data is somewhat confused by the rapid utilization of nitrous oxide but it appears that the isotope found its way to nitrous oxide most readily in the nitrous oxide adapted cells, and only after a lag in the nitrate adapted cells. The same pattern obtains in the conversion of nitrate to nitrogen. The failure to recover completely the N^{15} from nitrate in the nitrogen fraction is not explained although it may have been due to losses in sampling.

Repetition of this experiment under slightly altered conditions as to age of cells, concentration of substrate, and hydrogen acceptor yielded some-
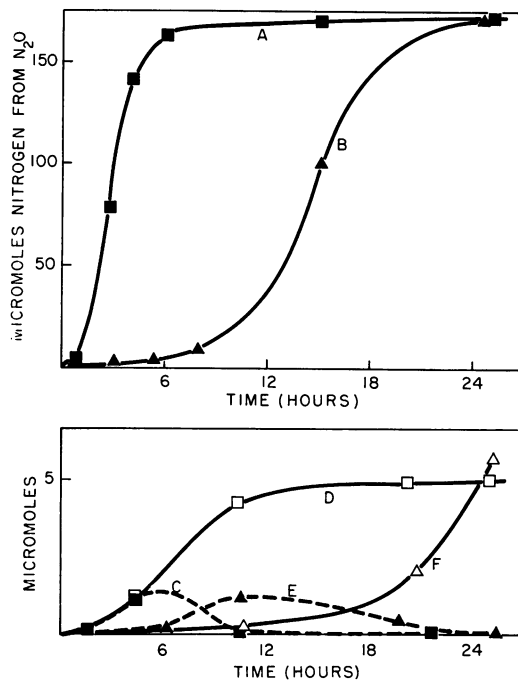

Figure 1. Comparison of cells grown on nitrous oxide and nitrate.

A (*Top*). Conversion of nitrous oxide to nitrogen by cells grown on nitrous oxide (curve *A*) and cells grown on nitrate ion (curve *B*). (*See* text.)

B (*Bottom*). Appearance of nitrate nitrogen in nitrous oxide (*broken lines*) and nitrogen (*solid lines*) with cells grown on nitrous oxide (curves *C* and *D*) and with cells grown on nitrate ion (curves *E* and *F*).

what different quantitative answers, but the general pattern observed in all experiments was the same. It was found, however, that the concentration of nitrate under which the cells were grown had considerable influence upon the length of the lag period.

To observe the effect of nitrate concentration, one set of cells was grown under conditions comparable with those of the preceding experiment, and another with one-fifth the concentration of nitrate. As can be seen by a reference to figure 2, a dependence of adaptation time on nitrate level was observed. The high nitrate cells exhibited a lag in the utilization of nitrous oxide and in the production of nitrogen gas, whereas with the low nitrate cells the lag period, although still discernible, was much shorter. There was an abundant production of gas under both sets of conditions while the cells were growing, so it seems unlikely
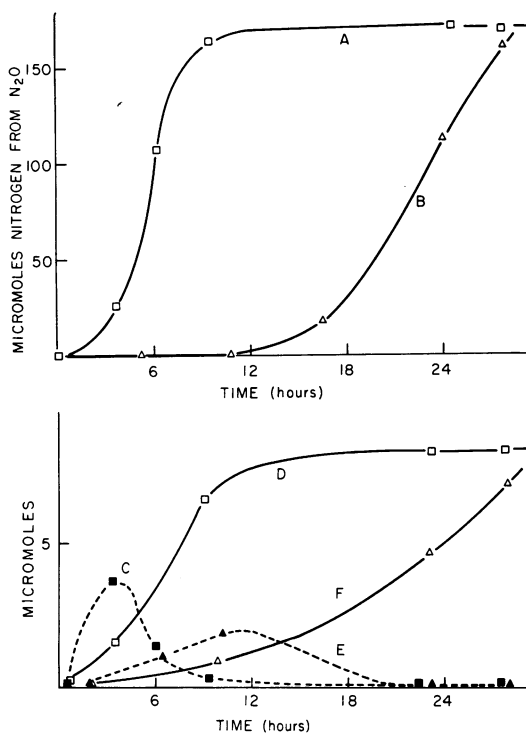
Figure 2. Comparison of two levels of nitrate ions.

A (Top). Conversion of nitrous oxide by cells grown on 0.01 molar nitrate ion (curve *A*), and cells grown on 0.05 molar nitrate ion (curve *B*).

B (Bottom). Appearance of nitrate nitrogen in nitrous oxide (*broken lines*) and nitrogen (*solid lines*) with cells grown on 0.01 M nitrate ion (curves *C* and *D*), and with cells grown on 0.05 M nitrate (curves *E* and *F*).

that the results of this experiment could be explained as due to nitrate production without further reduction in the case of the high nitrate cells with a complete reduction to nitrogen in the low nitrate cells. It would appear, therefore, that the only limitation on the high nitrate cells was in their ability further to reduce nitrous oxide without an adaptation period. No observations were made on the composition of gases produced during the growth period. A further comparison was made of the effect of adding all of the nitrate to the culture solution at the start as against adding it in increments roughly approximating the growth curve. The results of this procedure are shown in figure 3. As an additional comparison, oxygen grown cells were used for one set of tubes. Here the same general comparison could

be noted as was made between the high nitrate and low nitrate cells. When all of the nitrate (10 mmoles in 200 ml of culture solution) was added at the beginning of the growth period, the same lag in adaptation to nitrous oxide was observed, whereas with the cells to which nitrate had been added in increments, much less of a lag occurred and nitrous oxide was rapidly converted
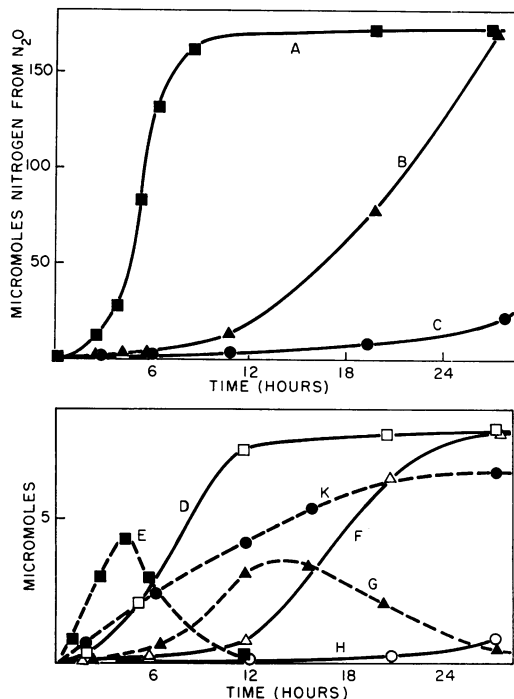

Figure 3. Effect of nitrate ion supplied in increments compared with nitrate supplied at beginning of growth period and compared with oxygen.

A (Top). Conversion of nitrous oxide to nitrogen by cells grown on nitrate ion added incrementally (curve *A*), nitrate ion supplied at beginning of incubation period (curve *B*), and oxygen (curve *C*).

B (Bottom). Appearance of nitrate ion in nitrous oxide (*broken lines*) and nitrogen (*solid lines*) by cells grown on nitrate ion added incrementally (curves *D* and *E*), nitrate ion added at beginning of growth period (curves *F* and *G*), and oxygen as hydrogen acceptor (curves *H* and *K*). For this incremental provision of nitrate ion 0.5 mmole of KNO_3 was added to the 200 ml of culture solution at the beginning of the incubation period, 1 mmole was added after 6 hr, 2.5 after 12 hr, and 6 after 18 hr for a total of 10 mmoles corresponding with the 0.05 M solution used in the other culture.

to nitrogen. Again, the ability to use nitrous oxide and the ability to adapt thereto was a function of the nitrate level in the culture solution. The higher the level of nitrate the longer the adaptation period required before the cells were able to utilize nitrous oxide.

N^{15} data for this experiment are presented in the lower portion of the figure ($3B$) and are consistent with the data on utilization of nitrous oxide. For cells grown under conditions of a sparse nitrate supply, the utilization of N^{15} nitrate and its conversion to nitrous oxide took place rapidly at first and upon the absorption of nitrous oxide and conversion to nitrogen, nitrous oxide containing N^{15} likewise disappeared from the medium.

The cells high in nitrate content also converted N^{15} nitrate to nitrous oxide but exhibited a considerable lag in this conversion. The same lag was observed in the conversion of N^{15} to nitrogen gas.

With oxygen grown cells, although a long lag was observed in their utilization of nitrous oxide and formation of nitrogen therefrom, the conversion of nitrate to nitrous oxide took place comparatively rapidly.

It is difficult to understand why these cells which had not been grown in the presence of nitrate still yielded a greater quantity of N^{15} to the nitrous oxide fraction than cells grown in the presence of 0.05 M nitrate. The most likely explanation is that the total production of nitrous oxide by the oxygen grown cells was no greater than that by the cells grown on 0.05 M nitrate but that the latter, possessing some capacity for nitrous oxide utilization, converted the nitrous oxide N^{15} to nitrogen and compounds intermediate between nitrous oxide and nitrogen sufficiently rapidly that not as much could escape from the reaction site to the solution and overlying atmosphere. Considering the possible compounds in which this isotopic nitrogen could be residing during this interval, the most likely ones are hyponitrite (or nitramide) and hydroxylamine or their respective enzymatically bound complexes, if such exist.

DISCUSSION

By using isotopically labeled nitrate or nitrite, it has been possible simultaneously to observe the conversion of these anions to nitrous oxide or nitrogen and the utilization of nitrous oxide. Besides the probably complicating effects of pH

(Verhoeven *et al.*, 1954) much of the conflicting information which has been accumulated on these phenomena can be explained on the basis of concentration of nitrate in the growth medium. Grown under low levels of nitrate, cells quickly adapt to the utilization of nitrous oxide, whereas under higher levels of nitrate, the adaptation to nitrous oxide takes place only after a considerable lag. Interpreted in terms of the earlier observation (Wijler and Delwiche, 1954) that the production of nitrous oxide was dependent upon nitrate concentration, the data suggest that the production of free nitrous oxide may occur under various conditions of denitrification but that its reutilization becomes of importance to the cells only where other hydrogen acceptors are not available. Under conditions of luxuriant supply of hydrogen acceptor in the form of nitrate, there is an abundant production of nitrous oxide but it is not reabsorbed extensively and nitrate is used preferentially as a hydrogen acceptor. When the supply of nitrate or nitrite is limited, nitrous oxide formed is reabsorbed and further denitrified to nitrogen gas. The failure simultaneously to adapt to nitrous oxide and nitrate by cells grown on nitrate is not in itself a justifiable argument for excluding nitrous oxide as an intermediate in the denitrification process. However, the data are highly suggestive and it is possible that nitrous oxide is formed in abundance only when nitrite or nitrate is present in sufficiently high concentrations that some subsequent reaction in the reduction sequence becomes limiting and a compound at the reduction level of hyponitrite is then split to produce nitrous oxide.

SUMMARY

Studies were made of the comparative rates of utilization of nitrous oxide by cells of *Pseudomonas denitrificans* grown on media containing nitrous oxide, oxygen, and various levels of nitrate ion as hydrogen acceptors, respectively.

A lag was observed in the utilization of nitrous oxide by cells grown in the presence of oxygen or of nitrate ion.

The length of the lag period observed was dependent upon the concentration of nitrate ion in the growth medium used. Low levels of nitrate ion result in a short lag period, and higher levels in a lag period considerably longer.

Cells grown on oxygen as a hydrogen acceptor exhibited a long lag period, in general longer than

that for nitrate although considerable variability was observed in this regard, perhaps due to the residual nitrate concentration of the medium used.

The possible significance of these observations is discussed.

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