SUBSTRATE OXIDATION AND NITROUS OXIDE UTILIZATION IN DENITRIFICATION

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It is generally assumed that during denitrification bacteria oxidize organic substances completely to carbon dioxide concomitant with the reduction of nitrate or nitrite to nitrogen and nitrous oxide. This assumption is based upon the early experiments of Gayon and Dupetit (1886) which demonstrated that the ratio of carbon dioxide to nitrogen in denitrifying cultures corresponded to the theoretical ratio to be expected for complete oxidation of the organic substrate. This evidence is not conclusive because a given ratio of carbon dioxide to nitrogen could result from either complete or incomplete oxidation of the substrate. In order to obtain unequivocal evidence on this point we have made complete carbon and nitrogen balances on a denitrifying culture in which succinate and glutamate were provided as organic substrates.

The role of nitrous oxide in denitrification is not clear, partly as a result of the use of inadequate techniques by earlier workers. Beijerinck and Minkman (1910) and Suzuki (1912) maintained that nitrous oxide was always present in the gaseous products of denitrification, whereas Gayon and Dupetit (1886) and others claimed that nitrous oxide was entirely absent in some of their experiments. Beijerinck and Minkman (1910) and Kluyver and Donker (1926) postulated that nitrous oxide is a normal precursor of nitrogen in denitrification, whereas later, Elema (1932) suggested a mechanism not involving nitrous oxide. We are reporting here a few observations which demonstrate that nitrous oxide is not always formed in denitrification, nor is it an obligatory intermediate in the conversion of nitrite to nitrogen.

METHODS

The organism used in this study was *Pseudomonas denitrificans*, which had been employed previously by Sacks and Barker (1949). The carbon-nitrogen balance experiment was done with 550 ml of a medium having the following composition per liter: sodium succinate·$6H_2O$, 4.90 g; KNO_3, 5.18 g; KH_2PO_4, 3.6 g; K_2HPO_4, 7.6 g; $MgSO_4 \cdot 7H_2O$, 0.27 g; $CaCl_2 \cdot 2H_2O$, 0.045 g; glutamic acid, 0.49 g; $FeCl_3 \cdot 6H_2O$, 0.0075 g; pH 7.3. The iron was added after autoclaving the medium.

The medium was placed in a completely filled fermentation flask and inoculated with 2 ml of a 48 hour culture grown on a medium having the following composition per liter: peptone, 4.0 g; KNO_3, 10.0 g; sodium succinate·$6H_2O$, 11.0 g; pH 7.2. The culture was incubated at 28 C. Evolved gas was collected over mercury and analyzed daily. The gas was shown to be essentially free of nitrous oxide by the method of Menzel and Kretzschmar (1929), modified to

include absorption of carbon dioxide in soda lime prior to combustion. Carbon dioxide was measured by absorption in 10 per cent KOH. The remaining gas was assumed to be N_2. Dissolved carbon dioxide was determined by the Van Slyke manometric method (Peters and Van Slyke, 1932). Total nitrogen was determined by a micro-Kjeldahl method, modified to include nitrate and nitrite (Pucher *et al.*, 1930). Residual glutamic acid was estimated by the chloramine T method (Cohen, 1940). Ammonia was determined by distillation from sodium

TABLE 1

Carbon and nitrogen balances for denitrification

COMPOUND	CARBON BALANCE	
	Before growth mg C	After growth mg C
Succinate	480	0
Glutamate	108	38
Carbon dioxide	—	496
Bacterial cells	—	43*
Total	588	577
Percentage recovery		98.1
	Nitrogen balance	
	mg N	mg N
Nitrate	394	2.0
Glutamate	25.8	8.9
Ammonia	—	6.4
Nitrogen gas	—	390
Bacterial cells	—	8.6†
Total	419.8	415.9
Percentage recovery		99.1

* Dry weight divided by 2.
† Dry weight divided by 10.

borate into boric acid and by titration. The cells were harvested by centrifugation and their dry weights determined after being washed.

Anaerobically grown cells for the manometric experiments were prepared as described previously (Sacks and Barker, 1949). The N_2O was obtained from the Ohio Chemical Company.

RESULTS

The carbon-nitrogen balance is shown in table 1. It is clear that succinate was mainly oxidized to carbon dioxide. About 8 per cent of the substrate carbon was converted into cell material. This is a somewhat greater carbon assimilation than occurs in most fermentations, but it is considerably less than that observed in many types of aerobic metabolism. The nitrogen evolved fully accounts for the nitrate-N; no N_2O could be demonstrated in the evolved gases.

Inasmuch as N_2O has been thought to occur universally in denitrification (Beijerinck and Minkman, 1910; Suzuki, 1912) and has been considered a normal intermediate in this metabolic system, we investigated the ability of cellular suspensions of denitrifying bacteria to utilize N_2O by the manometric technique. Vessels, with and without nitrite, having N_2 or N_2O atmospheres, were employed. Figure 1 gives the results of a single experiment. Figure 1a shows the relative utilization of N_2O in the presence and absence of nitrite. The ordinate values in figure 1 were calculated on the assumption that the production of nitrogen from nitrite was the only pressure producing process. This assumption is not strictly correct as applied to the vessels containing a nitrous oxide atmosphere.

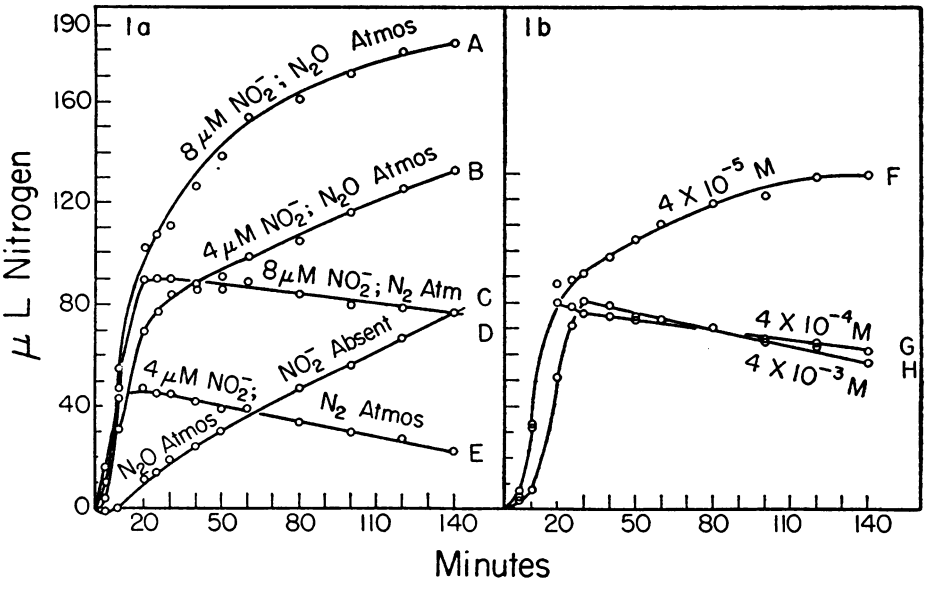

Figure 1a. The utilization of N_2O in the presence and absence of nitrite. All vessels contained 25 μM phosphate buffer, pH 7, and 40 μM sodium acetate in a total volume of 2 ml.

Figure 1b. The influence of azide on the utilization of N_2O. All vessels contained 25 μM phosphate buffer, pH 7, 40 μM sodium acetate, 8 μM sodium nitrite, and had N_2O atmospheres. Azide concentrations are shown on the figure.

The reduction of nitrous oxide to nitrogen will also cause a pressure increase due to the fact that nitrogen is less soluble in water than is nitrous oxide. The increase in pressure per mole of nitrogen formed in this process will be less than in the reduction of nitrite to nitrogen because of the simultaneous disappearance of nitrous oxide. Consequently, the rate of nitrogen formation from nitrous oxide is greater than that calculated directly from the pressure change.

Curve C of figure 1 indicates that nitrous oxide is reduced to nitrogen in the absence of nitrite. Comparison of curves A, B, and C shows that the presence of nitrite increases the rate of nitrous oxide utilization for 10 to 40 minutes following the time required for complete nitrite decomposition in the absence of nitrous oxide (curves D and E).

In several experiments, particularly those in which cellular suspensions were prepared from cultures over 40 hours old, there was a marked lag before the nitrous oxide was utilized, sometimes as much as 1 or 2 hours. This suggested the possibility that the enzymes required for the utilization of nitrous oxide were adaptive. To test this possibility, varying amounts of azide were added to vessels containing 8 micromoles of nitrite and an atmosphere of N_2O. Spiegleman (1947) has shown that azide inhibits the formation of adaptive enzymes. Comparison of curve A (no azide) [figure 1a] with curves F, G, and H (azide present in increasing concentrations) [figure 1b] shows that azide does not interfere with nitrite reduction but prevents the reduction of N_2O. Similar results were obtained in another experiment with dinitrophenol, which acts like azide (Monod, 1944). Although these results do not constitute proof of the adaptive nature of the N_2O reducing enzyme, since azide and dinitrophenol may be blocking the enzyme directly rather than inhibiting its formation, they do prove that nitrous oxide cannot be an obligatory intermediate in the formation of N_2 from nitrite.

The slow, linear pressure drop which occurred in vessels D, E, G, and H after the nitrite was exhausted is probably due to an uptake of traces of oxygen present in the vessels at the start of the experiment.

DISCUSSION

The carbon nitrogen balance experiment clearly establishes for the first time that an organic substrate for denitrification is completely oxidized to carbon dioxide, except for a small part that is converted to bacterial cells. The quantity of substrate assimilated is closer to that observed with anaerobic than with aerobic bacteria.

The absence of nitrous oxide in the balance experiment contradicts the conclusion of Beijerinck and Minkman (1910) that nitrous oxide is always produced, often as the chief product in denitrification. Gayon and Dupetit (1886) had claimed that *Bacterium denitrificans* β never produced N_2O whereas *Bacterium denitrificans* α produced it only when asparagine was present. Unfortunately, they did not record their analytical method for N_2O, forcing Beijerinck and Minkman to conclude that they probably used the electrical spark method with H_2, which is satisfactory only for gas mixtures containing more than 25 per cent N_2O. For the analysis of such mixtures Beijerinck and Minkman (1910) combusted with a known volume of hydrogen in the Drehschmidt platinum capillary.

Suzuki (1912) reinvestigated the problem and criticized Beijerinck and Minkman's technique of preliminary removal of oxygen (which apparently was always present in some degree) by reaction with phosphorus. Suzuki maintained that the phosphorus did not remove O_2 completely and did react with N_2O to some extent. The technique employed by Suzuki consisted of preliminary removal of oxygen with pyrogallol, carbon dioxide with KOH, and finally reduction in the Drehschmidt platinum capillary with CO_2-free carbon monoxide. The carbon dioxide produced was subsequently absorbed in alkali. He carried out extensive experiments to determine the N_2O lost by solution in pyrogallol and KOH, and concluded such losses were not appreciable when the partial pressure of N_2O was

low. Using this technique he corroborated the claim of Beijerinck and Minkman that N_2O was always present in denitrifying cultures, although his work indicated that considerably less N_2O was produced than in comparable cultures of the preceding workers. However, in all the pure culture experiments of Suzuki, the concentration of KNO_3 was never less than 10 per cent; only in enrichment cultures was the concentration as low as 0.2 per cent. It is well known that a high concentration of nitrate favors nitrous oxide accumulation.

The method used for N_2O analysis in the present work appears unobjectionable at present. Preliminary absorption with soda lime removes CO_2 which otherwise would be reduced to carbon monoxide (Treadwell and Hall, 1937) and the N_2O is reduced with hydrogen in a platinum capillary. Menzel and Kretzschmar (1929) demonstrated the method to be accurate when the N_2O varied from 0.4 per cent to 100 per cent. The amount of nitrous oxide, if any, in the carbon-nitrogen balance experiment must have been less than 0.4 per cent of the total gas.

Although nitrous oxide did not accumulate, the possibility of its occurrence as an intermediate was considered. Earlier workers (Beijerinck and Minkman, 1910; Kluyver and Donker, 1926) had postulated that nitrous oxide is a stage in the reduction of nitrite to molecular nitrogen, but the evidence presented for this hypothesis was indirect, and no critical supporting evidence has been brought forward subsequently. The evidence presented here indicates that nitrous oxide cannot be a normal intermediate in the formation of nitrous oxide because: (1) the utilization of nitrous oxide by resting cells may be selectively blocked by azide or dinitrophenol under conditions which permit the formation of nitrogen from nitrite at normal rates; and (2) a lag frequently precedes the utilization of N_2O by resting cells, so that nitrite may be reduced to nitrogen during a period when nitrous oxide is not being converted to nitrogen.

These facts indicate that nitrous oxide is formed by a side reaction. Hyponitrite, a possible intermediate in denitrification, might conceivably be converted to either N_2 or N_2O, depending on the organism and the environmental conditions.

SUMMARY

A carbon nitrogen balance is given for *Pseudomonas denitrificans* growing under denitrifying conditions in a medium of known composition. It is demonstrated that succinate, the hydrogen donor, is completely oxidized to carbon dioxide, while nitrate is reduced to nitrogen. No nitrous oxide accumulates.

Denitrifying resting cells of *P. denitrificans* can reduce N_2O to N_2, but a lag period is often observed before the conversion commences. Suitable concentrations of azide and dinitrophenol inhibit the utilization of N_2O without affecting the reduction of nitrite to N_2. This is considered evidence that N_2O cannot be the normal precursor of N_2 in the reduction of nitrite to N_2.

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