# The Effect of Nitrous Oxide on Biological Nitrogen Fixation and the Uptake of Combined Nitrogen

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Some years ago it was found in this laboratory [1] in long time growth experiments with *Azotobacter vinelandii* that $N_2O$ inhibits not only the fixation of molecular nitrogen but also the utilization of nitrate nitrogen. The utilization of ammonium nitrogen was not inhibited. Later Mozen *et al.*[2] observed in short time experiments with the same strain of *A. vinelandii*, using nitrate labelled with $^{15}N$, that the utilization of nitrate nitrogen was not inhibited by $N_2O$. In the last mentioned experiments the cells were harvested 3—24 h after inoculation. The reason for this difference between long time and short time experiments is unexplained. I have controlled the previous results from long time growth experiments with *A. vinelandii* harvesting the cells 48 and 144 h after inoculation. The results of the experiments are presented in this paper. The experiments were performed with the same strain

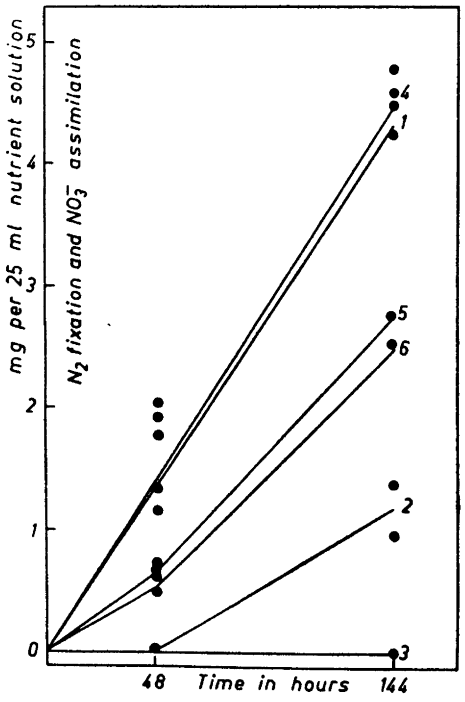


*Fig. 1.* The effect of $N_2O$ on $N_2$ fixation and $NO_3^-$ assimilation by *Azotobacter*.

| Curve | Gas-mixture | | | N source |
|---|---|---|---|---|
| | $N_2$ | $O_2$ | $N_2O$ | |
| 1 | 0.2 | 0.2 | — | $N_2$ |
| 2 | 0.2 | 0.2 | 0.6 | $N_2$ |
| 3 | — | 0.2 | 0.8 | — |
| 4 | 0.2 | 0.2 | — | $NO_3^-$ |
| 5 | 0.2 | 0.2 | 0.6 | $NO_3^-$ |
| 6 | — | 0.2 | 0.8 | $NO_3^-$ |

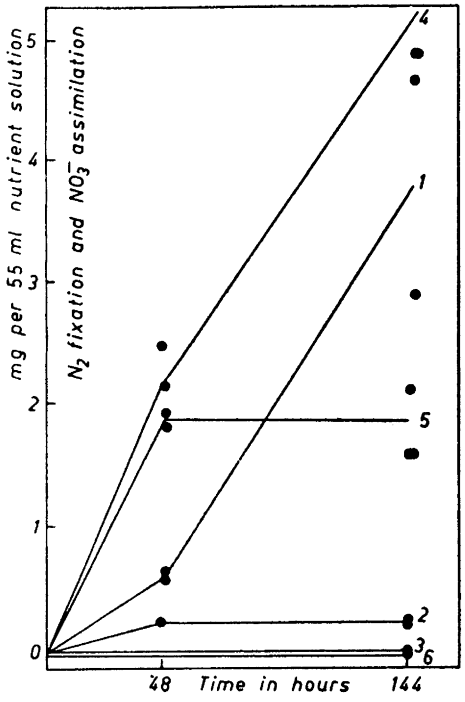


*Fig. 2.* The effect of $N_2O$ on $N_2$ fixation and $NO_3^-$ assimilation by *Clostridium*.

| Curve | Gas-mixture | | N source |
|---|---|---|---|
| | $N_2$ | $N_2O$ | |
| 1 | 1.0 | — | $N_2$ |
| 2 | 0.4 | 0.6 | $N_2$ |
| 3 | — | 1.0 | — |
| 4 | 1.0 | — | $NO_3^-$ |
| 5 | 0.4 | 0.6 | $NO_3^-$ |
| 6 | — | 1.0 | $NO_3^-$ |

*Table 1.* The effect of $N_2O$ on $N_2$ fixation by *Azotobacter vinelandii* K.

$N_2$ fixation mg N/25 ml nutrient solution

| Gas-mixtures | 0.2 atm. $N_2$ 0.2 atm. $O_2$ | | 0.2 atm. $N_2$ 0.2 atm. $O_2$ 0.6 atm. $N_2O$ | | 0.2 atm. $O_2$ 0.8 atm. $N_2O$ | |
|---|---|---|---|---|---|---|
| Inoculum + nutrient | Tot. N in cells | Increase of N | Tot. N in cells | Increase of N | Tot. N in cells | Increase of N |
| | 0.130 | | 0.130 | | 0.130 | |
| 48 hours | 1.317 | 1.187 | 0.195 | 0.065 | 0.100 | — |
| | 1.934 | 1.804 | 0.154 | 0.024 | 0.160 | 0.030 |
| 144 hours | 4.251 | 4.121 | 1.512 | 1.382 | 0.131 | — |
| | 4.799 | 4.669 | 1.080 | 0.950 | — | — |

of *A. vinelandii* as earlier. Similar experiments were also performed with *Clostridium pasteurianum.*

*Azotobacter* was incubated at 30°C in Burk's medium containing 10 p.p.m. Mo, and *Clostridium* was incubated at 37°C in synthetic nutrient solution containing the essential vitamins [3].

The inocula for *Azotobacter* were grown for 24 h, aerating the culture very heavily. The inocula for nitrate culture were adapted thrice in the basal nutrient solution containing 500 p.p.m. nitrate nitrogen as $KNO_3$.

The inocula for *Clostridium* were grown for 36 h and the culture was enriched with nitrogen, bubbling a nitrogen-stream continually through the culture. The nitrate inocula were adapted in a similar way as by *Azotobacter.*

The experiments were carried out in six vacuum desiccators each of 8 l. Each desiccator contained 4 bottles of 100 ml each containing 25 ml nutrient solution. Gas-mixtures: *Azotobacter:* 0.2 atm. $N_2$ and 0.2 atm. $O_2$, 0.2 atm. $N_2$, 0.2 atm. $O_2$ and 0.6 atm. $N_2O$, and 0.2 atm. $O_2$ and 0.8 atm. $N_2O$. *Clostridium:* 1.0 atm. $N_2$, 0.4 atm. $N_2$ and 0.6 atm. $N_2O$, and 1.0 atm. $N_2O$.

In order to get the gas-mixtures into the desiccators the treatment of *Azotobacter* desiccators was: vacuum, flushing with air, filling with the required gas-mixture; and of *Clostridium* desiccators: vacuum, flushing with nitrogen twice, filling with required gas-mixtures.

48 and 144 h after inoculation two bottles from each desiccator were taken for analyses. The samples were immediately put on ice, centrifuged and the supernatant was used for nitrate analysis (bottles with nitrate). The

*Table 2.* The effect of $N_2O$ on $NO_3^-$ assimilation by *Azotobacter vinelandii* K.

Utilization of $NO_3^-$ mg/25 ml nutrient solution

| Gas-mixtures | 0.2 atm. $N_2$ 0.2 atm. $O_2$ | | | | 0.2 atm. $N_2$ 0.2 atm. $O_2$ 0.6 atm. $N_2O$ | | | | 0.2 atm. $O_2$ 0.8 atm. $N_2O$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Inoculum + nutrient | Tot. N in cells | Increase of N | $NO_3$-N in sol. | $NO_3$-N used | Tot. N in cells | Increase of N | $NO_3$-N in sol. | $NO_3$-N used | Tot. N in cells | Increase of N | $NO_3$-N in sol. | $NO_3$-N used |
| | 0.397 | | 18.90 | | 0.397 | | 18.90 | | 0.397 | | 18.90 | |
| 48 hours | 1.771 | 1.374 | 17.42 | 1.48 | 1.060 | 0.663 | 18.23 | 0.66 | 1.096 | 0.699 | 18.17 | 0.73 |
| | 2.488 | 2.091 | 16.75 | 2.15 | 1.080 | 0.683 | | | 0.990 | 0.593 | 18.37 | 0.51 |
| | 2.360 | 1.963 | | | 1.139 | 0.742 | 18.10 | 0.79 | | | | |
| 144 hours | 4.776 | 4.379 | 14.57 | 4.33 | 3.14 | 2.743 | 16.12 | 2.78 | 2.782 | 2.385 | | |
| | 4.847 | 4.450 | 14.50 | 4.40 | | | 16.20 | 2.71 | 2.963 | 2.566 | 16.40 | 2.51 |

*Table 3.* The effect of $N_2O$ on $N_2$ fixation by *Clostridium pasteurianum* W 5.

$N_2$ fixation mg/55 ml nutrient solution

| Gas-mixtures | 1.00 atm. $N_2$ | | 0.4 atm. $N_2$ 0.6 atm. $N_2O$ | | 1.00 atm. $N_2O$ | |
|---|---|---|---|---|---|---|
| Inoculum + nutrient | Tot. N in cells | Increase of N | Tot. N in cells | Increase of N | Tot. N in cells | Increase of N |
| | 0.526 | | 0.526 | | 0.526 | |
| 48 hours | 1.136 | 0.610 | 0.775 | 0.249 | 0.504 | — |
| | 1.050 | 0.524 | 0.429 | — | 0.492 | — |
| 144 hours | 5.137 | 4.611 | 0.715 | 0.189 | 0.512 | — |
| | 3.388 | 2.864 | 0.698 | 0.172 | 0.461 | |

cells were washed once with distilled water and a Kjeldahl-analysis was made from the washed cells. The increase of nitrogen in the cell mass and the decrease of nitrate in the solution correspond well with each other in the *Azotobacter* experiments, wherefore analytical errors have not influenced the results essentially. In *Clostridium* experiments the increase of nitrogen in the cells is somewhat larger than the decrease of nitrate in the solution, and hence a slight $N_2$ fixation might have occurred even when nitrate formed the source of nitrogen.

Tables 1 and 2 and Fig. 1 present nitrogen fixation and the utilization of nitrate nitrogen by *Azotobacter*. During 48 h practically no $N_2$ fixation could be found in *Azotobacter* cultures with 0.6 atm. $N_2O$. After 144 h some $N_2$ fixation was, strangely enough, found under these conditions (Fig. 1, curves 1, and 2). The inhibition of the utilization of nitrate nitrogen is clear already 48 h after inoculation and especially after 144 h at both $N_2O$ concentrations (Fig. 1, curves 4, 5, and 6).

The results with *Clostridium* are presented in Tables 3 and 4, and in Fig. 2. In *Clostridium* cultures 1.0 atm. $N_2O$ totally prevented nitrate utilization after 48 h. 48 h after inoculation the utilization of $NO_3$-N was about 1 mg/55 ml nutrient solution (Tables 3 and 4). The inhibiting effect of nitrous oxide on the utilization of nitrate in long time growth experiments is difficult to explain. Since it is not met with in short time experiments, as is the case with molecular nitrogen fixation, in long time growth experiments we probably have to do with some secondary influence which is not known more closely.

The experimental part of the work was performed in the autumn of 1954.

*Table 4.* The effect of $N_2O$ on $NO^-$ assimilation by *Clostridium pasteurianum* W 5.

Utilization of $NO_3^-$ mg/55 ml nutrient solution

| Gas-mixtures | 1.00 atm. $N_2$ | | | | 0.4 atm. $N_2$ 0.6 atm. $N_2O$ | | | | 1.00 atm. $N_2O$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Inoculum + nutrient | Tot. N in cells | Increase of N | $NO_3$-N in sol. | $NO_3$-N used | Tot. N in cells | Increase of N | $NO_3$-N in sol. | $NO_3$-N used | Tot. N in cells | Increase of N | $NO_3$-N in sol. | $NO_3$-N used |
| | 0.759 | | 10.94 | | 0.759 | | 10.94 | | 0.759 | | 10.94 | |
| 48 hours | 2.94 | 2.181 | 9.16 | 1.78 | 2.56 | 1.801 | 9.95 | 0.99 | 0.720 | — | 10.85 | — |
| | 3.25 | 2.491 | 8.95 | 1.99 | 2.70 | 1.941 | 9.90 | 1.04 | 0.735 | — | 10.99 | — |
| 144 hours | 5.61 | 4.851 | 7.96 | 3.98 | 2.32 | 1.561 | 10.07 | 0.87 | 0.691 | — | 10.47 | — |
| | 6.09 | 5.331 | 6.77 | 4.17 | 2.34 | 1.581 | 9.99 | 0.95 | 0.701 | — | 10.05 | — |
| | 5.61 | 4.851 | | | 2.95 | 2.191 | | | | | | |

1. Virtanen, A. I. and Lundbom, S. *Acta Chem. Scand.* **7** (1953) 1223.
2. Mozen, M. M., Burris, R. H., Lundbom, S. and Virtanen, A. I. *Acta Chem. Scand.* **9** (1955) 1232.
3. Virtanen, A. I. and Lundbom, S. *Acta Chem. Scand.* **8** (1954) 870.

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# On the Rearrangement Products of Humulone

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The main bitter substances of beer are the *iso*humulones formed by rearrangement of the humulone, cohumulone and adhumulone of hops during the wort boiling. They have been isolated from trimethylpentane extracts of beer by countercurrent distribution [1]. According to Windisch *et al*, however, two products were formed from humulone when rearranged in a buffer solution, as the preformed *iso*humulone (Harzkörper A) was in part degraded to a second compound of similar properties (Harzkörper B)[2]. The latter compound and its analogues have not been observed in beer. When treated with boiling alkaline ethanol humulone is stated to give *iso*humulone only[3].

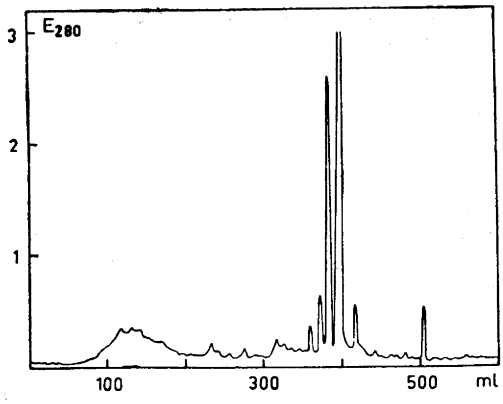


*Fig. 1.* Distribution diagram for rearrangement products of humulone in an acetate buffer of pH 5.1. The last peak is unreacted humulone.

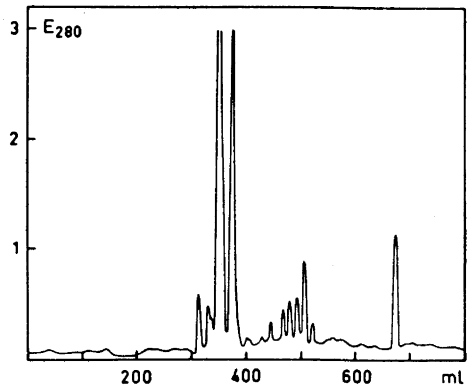


*Fig. 2.* Chromatographic pattern of the rearrangement products of humulone from alkaline ethanol.

We have now tried to study humulone rearrangement products of different origins by reversed-phase partition chromatography. The separations were performed on 800 × 8 mm columns of hydrophobic Hyflo Super Cel with chloroform as the stationary solvent. Gradient elution was accomplished with a buffer solution containing 25% methanol, the pH-value of which was steadily increased. The optical density of the elute was measured continuously by a spectrophotometer connected to a recorder [4].

When humulone was rearranged at 100°C in a number of buffer solutions of medium pH-value two principal components were always found, which were eluted at pH 5.8—6.0 (Fig. 1). Their ultraviolet absorption spectra were similar to that of the *iso*humulones of beer and to that of humulinic acid. It has been shown earlier [4] that the chromatographic pattern of the bitter substances of beer was predominated by a five (or six) peaks appearing at pH 5.6—6.2. It seemed probable that the group consisted of six components, two from each of the three humulones.

These results have led us to assume that the two same principal compounds are formed from humulone in beer and in buffer solutions of medium pH-value. A detailed study of the humulone rearrangements in buffer solutions thus is to be desired.