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Anaerobic Nitrogen Fixation and Formation of Oxime Nitrogen

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Nitrogen fixation of greatest significance takes place, in nature, in the leguminous root nodules. The legume bacteria are strongly aerobic and the oxygen supply in the root nodules is assured by a special hemoglobin, leghemoglobin, which transports and stores oxygen. In nodules lacking this chromoprotein, no nitrogen fixation has been detected.

Of the free-living nitrogen fixing bacteria, *Azotobacters* are the most rapid and efficient nitrogen-fixers. They, too, are strongly aerobic and respirate intensely.

In nitrogen fixation by Azotobacters and leguminous root nodules, the formation of oxime nitrogen has been noted. Attention must be paid to this fact in explaining the mechanism of nitrogen fixation, and this has given rise to doubt as to whether nitrogen fixation is a pure reduction, occurring, for instance, in the following way (1) acc. to Wieland.

$$N_2 \xrightarrow{H_2} HN = NH \xrightarrow{H_2} H_2N{-}NH_2 \xrightarrow{H_2} NH_3 \qquad (1)$$

Oxime formation in connection with reaction (1) could be explained by assuming that the first reduction product, the di-imide, combines with water whereby hydroxylamine is produced (2), but there is no proof for this kind of reaction.

$$N_2 \xrightarrow{H_2} HN = NH \xrightarrow{H_2O} NH_2OH \qquad (2)$$

Blom believed that hydration of the nitrogen molecule was the first reaction in nitrogen fixation. Reduction of this intermediate would lead to hydroxylamine (reaction 3).

$$N_2 \xrightarrow{H_2O} \begin{matrix} HN & - & NH \\ | & & | \\ HO & & OH \end{matrix} \xrightarrow{H_2} NH_2OH \qquad (3)$$

Later, Virtanen [1] introduced a quite different possibility for the formation of oxime nitrogen. According to this theory the first reaction in nitrogen fixation is oxidation. The transfer of electron from the nitrogen atom to oxygen would be effected by a hemin system as in respiration. The hydration and reduction of nitrous oxide would then lead to hydroxylamine (reaction 4).

$$N_2 \longrightarrow N \longrightarrow N^+ \xrightarrow{O^{--}} N_2O \xrightarrow{H_2O} (NOH)_2 \xrightarrow{H_2} NH_2OH \qquad (4)$$

There is still another route to the formation of hydroxylamine, *viz.*, the oxidation of ammonia. In the nitrification caused by specific bacteria, ammonia is known to be oxidized to nitrite and nitrate. Eggleton [2], Pearsall and Billimoria [3] have concluded that ammonia is also oxidized in green plants. The formation of hydroxylamine from ammonia was noted by Steinberg [4] in cultures of *Aspergillus*. Azotobacter also forms oxime nitrogen from ammonia [5]. If oxime nitrogen arises even in the nitrogen fixation, via ammonia oxidation, hydroxylamine would not be an intermediate and the formation of oxime would in no way invalidate the reaction (1) even if reactions 2 and 3 would not exist.

Virtanen and Miss Järvinen have examined the formation velocity of oxime nitrogen in *Azotobacter* cultures grown in nutrient solutions with either ammonium nitrogen, nitrate nitrogen, or molecular nitrogen as the nitrogen source. Equal amounts of *Azotobacter vinelandii* suspensions were added to each solution. Because of the large number of bacteria, a detectable increase in the nitrogen content occured already after about an hour, and the oxime formation could be followed over a short period. Where the molecular nitrogen and nitrate nitrogen served as the source of nitrogen, oxime nitrogen was detectable much sooner in the cells and solutions than when ammonium phosphate was employed. In general, oxime nitrogen could be found in cells grown on N_2 and NO_3^- already after 60—90 mins., but on NH_4^+ not until after 150—180 min. The cells assimilated ammonium nitrogen as rapidly as or more rapidly than molecular nitrogen. Assimilation of nitrate was slowest. Thus these experiments, which will be reported in detail in another paper, do not support the concept that oxime nitrogen is formed from ammonia in nitrogen fixation by Azotobacters. Hence, reactions 2, 3 or 4 would demand the most attention when interpreting the formation of oxime nitrogen.

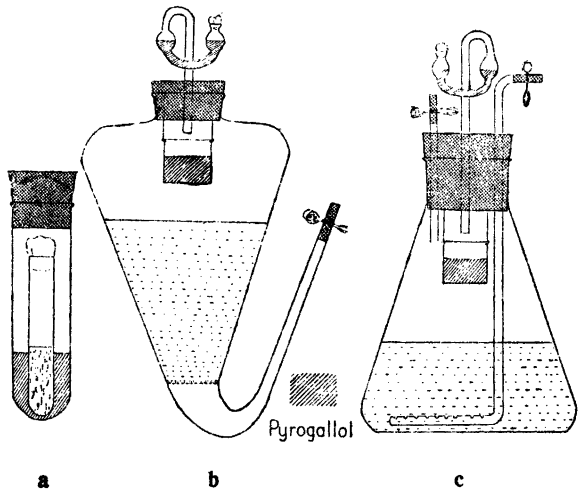

Fig. 1. Apparatuses used for experiments on anaerobic nitrogen fixation.

To ascertain if reactions 2 and 3 may be concerned in the formation of oxime nitrogen, we have examined whether or not oxime nitrogen is produced in fully anaerobic nitrogen fixation. Reaction 4 is, under anaerobic conditions, out of the question. In the following we record our experiments.

EXPERIMENTAL

The culture of *Clostridium butyricum* was obtained from Delft Technische Hogeschool through the courtesy of Prof. A. J. Kluyver. An anaerobic bacterium of the same type was isolated by the authors from the soil. Both organisms fix nitrogen and yield, among other fermentation products, hydrogen and butyric acid. The organism isolated by the authors was called *Clostridium X*. It was isolated in the following manner: 2.5 g leaf mould were suspended in 10 ml water and kept for 20 min at 80° C. 5 ml of the heated suspension were placed in a test tube containing nutrient solution free from combined nitrogen. As soon as a distinct gas evolution was observed 1 ml of the suspension was inoculated into a new nutrient solution (10 ml). The procedure was repeated 6—7 times in succession. Anaerobic conditions were maintained in the test tubes. The lack of combined nitrogen and the anaerobic conditions guaranteed that only the anaerobic fixers were enriched. A microscopically homogeneous culture was obtained.

Nutrient solution and growth conditions. The composition of the nutrient solution was: 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, 0.1 g $CaSO_4$, 0.01 g $Fe_2(SO_4)_3$, 0.0252 mg Na_2MoO_4, 10 g $CaCO_3$, 1 l. water. The pH of the solution was 7.3—7.4. The solution resembles that prescribed by Burk [6] for Azotobacter, containing in addition 0.01 p. p. m. Mo, which, according to Jensen [7], promotes optimally nitrogen fixation by Cl. butyricum. $CaCO_3$ was added to neutralize the acids formed in fermentation. The solution contained 1.51 mg N per litre. The temperature in every experiment was about 30°.

In order to establish anaerobic conditions, an alkaline pyrogallol solution was employed in cultivating the bacterium. The pyrogallol solution was kept in a wide test tube closed with a rubber stopper and inside this was placed a test tube closed with cotton wool and containing the bacterial culture (Fig. 1a).

The fermentation experiments proper were carried out in 2 litre Kluyver flasks. The volume of the nutrient solution varied from 1000 to 1250 ml. Anaerobic conditions were accomplished by leading nitrogen gas, which had first passed through two wash bottles filled with alkaline pyrogallol solution through the solution. During sterilization, the Kluyver flask was closed with cotton. The rubber stopper and accessories were sterilized separately and then the cotton replaced by it. The whole system (Fig. 1b) was kept in water at 29—30°. During the experiment, nitrogen gas was occasionally passed through the solution to mix it.

In two experiments a slightly different procedure was employed. A 750 ml wide-mouthed Erlenmeyer flask containing 500 ml nutrient solution was used as a container. This system is illustrated by Fig. 1c.

Determination of oxime nitrogen. Oxime nitrogen was determined according to Blom [8] and Endres[9]. Csáky [10] found that only the > C:NOH group will be determined by this method. Since the concentration of oxime nitrogen could be so low that the reaction for oxime would be negative, the solution was concentrated in many experiments by vacuum distillation. Determination of oxime nitrogen was made from both the original and the concentrated solutions, as can be seen from Table 1.

Table 1. Formation of oxime nitrogen in anaerobic nitrogen fixation.

Organism	Growth days	Nutrient solution		Culture solution at the end of the experiment		Nitrogen fixed		Oxime nitrogen in	
		sugar g/l	nitrogen mg/l	sugar g/l	nitrogen g/l	mg/l	mg/g glucose	culture solution	conc. culture solution
Cl. butyricum	7	9.36	1.51	7.7	5.32	3.81	2.3	0	0 [1]
Cl. X.	9	9.36	1.51	5.1	14.6	13.1	3.1	0	0 [2]
Cl. X.	5	9.36	1.51	0	29.7	28.2	3.0	0	0 [3]
Cl. X.	4	9.36	1.51	4.9	8.8	7.3	1.6	0	0 [4]
Cl. X.	6	9.36	1.51	0	14.1	12.6	1.3	0	0

In one experiment, nitrogen was determined also in the clear centrifugate in order to ascertain the quantity of fixed nitrogen in the cells and in the solution (Table 2).

Table 2. Occurrence of anaerobically fixed nitrogen in nutrient solutions outside cells.

Organism	Growth days	N in nutr.soln. mg/l	Final N in culture soln. mg/l	N in clear centrifugate mg/l	Fixed N in solution %
Cl. X.	6	1.51	14.1	9.07	60

[1] Concentrated to 1/20. [2] 1/10. [3] 1/37. [4] 1/14.

In addition to the experiments described above, another experiment was made with *Clostridium X* in a Kluyver flask containing 99.3 vol. % N_2 and 0.7 vol. % O_2. Clostridium grew still well and used sugar as well as in fully anaerobic conditions. Table 3 shows the decrease of glucose and the formation of oxime in this experiment.

Table 3. Experiment with Clostridium X in low oxygen tension (0.7 vol. % O_2).

Growth, days	Glucose mg/ml	Oxime nitrogen	
		in culture solution	in conc. culture solution
0	20	0	—
3	16.8	0	—
6	9.2	0	—
9	3.3	0	0

Accordingly, *Clostridium* did not form oxime-N even if some oxygen was present. The result suggests that an anaerobic N-fixer is unable to form oxime nitrogen even in low oxygen concentration.

RESULTS AND CONCLUSIONS

The data concerning nitrogen fixation and formation of oxime nitrogen in fully anaerobic nitrogen fixation are summarized in Table 1. As can be seen no traces of oxime nitrogen could be detected even in very concentrated solutions by the sensitive method of Blom. In corresponding experiments with *Azotobacter*, the authors have always found considerable amounts oxime nitrogen. Oxime nitrogen can, in this case, be determined directly from the growth solution, without concentration. Consequently, our results with anaerobic *Clostridium* suggest that oxime nitrogen is not formed in nitrogen fixation under anaerobic conditions although the possibility exists that the oxime formed is reduced too rapidly to be detected. But such a possibility exists likewise in the aerobic nitrogen fixation. Since, moreover, oxime nitrogen was not formed in the *Clostridium* culture even in low oxygen tension (Table 3) formation of oxime nitrogen, according to reactions 2 and 3, whereby hydroxylamine would arise on addition of water to di-imide or nitrogen molecule, does not seem likely. On the other hand, the results obtained justify the conclusion that nitrogen fixation takes place anaerobically via reduction. Whether di-imide and hydrazin are hereby formed as intermediates acc. to reaction 1 is questionable.

The formation of oxime nitrogen, regularly noted in aerobic N-fixation, implies that the first phase in aerobic nitrogen fixation would be oxidative (reaction 4) or that oxime nitrogen arises from hydroxylamine formed from ammonia through oxidation. As was mentioned in the beginning of this paper, the

observations on the formation velocity of oxime nitrogen do not fit easily into the latter concept attractive though it might seem. Provided that oxime nitrogen in aerobic nitrogen fixation could later be explained to result from ammonia oxidation there would be no more objection to assume that nitrogen fixation takes place purely reductively in both aerobic and anaerobic conditions. At the present moment, oxime formation cannot be explained without by-hypotheses in this way.

The difference between the mechanisms of aerobic and anaerobic nitrogen fixation is suggested also by the fact that gaseous hydrogen prevents aerobic but probably not anaerobic nitrogen fixation because H_2 is formed in the fermentation of sugar by *Cl. butyricum*.

SUMMARY

In the anaerobic nitrogen fixation by *Clostridium butyricum* and by another bacterium of the same type, no traces of oxime nitrogen could be found. In the aerobic nitrogen fixation by *Azotobacter* oxime nitrogen is always formed. Theoretical conclusions of the course of nitrogen fixation and of the formation of oxime nitrogen are drawn on the basis of this finding.

Clostridium does not form oxime nitrogen even in low oxygen tension. When the atmosphere contained 0.7 vol. % O_2 and 99.3 vol. % N_2 *Clostridium* still grew well but no traces of oxime-N could be detected.

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