ACTA CHEMICA SCANDINAVICA 5 (1951) 220–226

On the Formation of Bound Hydroxylamine in *Azotobacter*

ARTTURI I. VIRTANEN and HELVI JÄRVINEN

Laboratory of the Foundation for Chemical Research, Biochemical Institute, Helsinki, Finland

The formation of hydroxylamine as an intermediate in *Azotobacter* cultures is very plausible both when molecular nitrogen and nitrate nitrogen serve as the source of nitrogen. A compound is then regularly formed in which an NOH-group is linked with a carbon atom. By hydrolyzing such compounds with sulphuric acid and by oxidizing then with iodine, nitrite is produced[1,2]. Consequently, the intermediate would contain either oxime, $>C=NOH$-group, or hydroxamic acid, $-C\overset{O}{\underset{NHOH}{<}}$-group. We have regarded the former as likely but the latter, too, must undoubtedly be considered, especially since Speck[3] and Elliot[4] discovered that an enzyme system acting in the synthesis of glutamine from glutamic acid and ammonia in animal tissues also causes formation of hydroxamic acid from glutamic acid and hydroxylamine. Recently Waelsch *et al.*[5] and Grossowicz *et al.*[6] have noted in bacteria and Stumpf and Loomis[7] in plants another interesting enzymatic reaction which also leads to hydroxamic acid:

$$\text{glutamine} + NH_2OH \rightarrow \text{glutamohydroxamic acid} + NH_3$$

In addition to this system hydroxylamine also reacts with asparagine forming the corresponding hydroxamic acid.

The present investigation which was carried out more than a year ago (preliminary report[8]) does not deal more closely with the question whether the hydroxylamine, which is set free in the hydrolysis of Azotobacter, originates from oxime or hydroxamic acid or from both of them. This question is now under investigation in this laboratory. From the point of view of nitrogen cycle it is most important that the formation of either compound is probably preceded by formation of hydroxylamine which thus would be a common intermediate both in nitrate reduction and nitrogen fixation[9].

Since, however, bound hydroxylamine is also formed when ammonium salts serve as the source of nitrogen for *Azotobacter* it is not impossible that hydroxylamine may be produced by oxidation from ammonia, and hence, its formation in the nitrogen fixation and nitrate reduction should not be a proof of its intermediary nature.

In order to obtain elucidation to this important theoretical question we examined the formation velocity of bound hydroxylamine in *Azotobacter* cultures by using in parallel experiments N_2, NO_3^- and NH_4^+ for the source of nitrogen. In order to speed up assimilation of nitrogen and thus to enable analytical determination of oxime nitrogen in experiments of short duration we used so heavy inoculation of *Azotobacter* in most experiments that nitrogen fixation as well as assimilation of other nitrogen sources was analytically detectable within 1—2 hours. In the following some characteristic experiments are recorded both from experiments of long duration (normal inoculation) and experiments of short duration (heavy inoculation).

EXPERIMENTAL

Azotobacter vinelandii (strain Lipmann, original culture obtained from Prof. A. J. Kluyver in Delft some years ago) was cultivated in a nutrient solution containing K_2HPO_4 1.6 g, KH_2PO_4 0.4 g, $MgSO_4$ 0.2 g, NaCl 0.2 g, $CaSO_4$ 0.1 g, $Fe_2(SO_4)_3$ 0.01 g, $FeSO_4$ 0.015 g, glucose 5 g, saccharose 20 g, Na_2MoO_4 0.003 g, tap water 1 000 g. pH of the nutrient solution was 7.2.

Nitrogen liberated in the hydrolysis as hydroxylamine (*bound hydroxylamine*) was determined according to the principle of Endres by hydrolyzing for 6 h with 3 *N* sulphuric acid, by oxidizing the hydroxylamine set free with iodine to nitrite and by determining nitrite (Blom [1]). In details the procedure of Csáky [10] was followed.

Total N was determined by the micro method of Miller [11]. The number of bacteria was counted by the Skar ocular (Virtanen [12]).

Experiments of long duration by using small inoculation

In the first experiments bound hydroxylamine-N was determined when the above mentioned nutrient solution either without combined nitrogen or with NH_4^+ or NO_3^- was inoculated with a small amount of Azotobacter culture. 5 ml of 2 days old bacterial culture without combined nitogen was inoculated into 200 ml of nutrient solution in 1 litre flat flasks, one without combined nitrogen, one containing 40 mg % ammonium nitrogen and one 40 mg % nitrate nitrogen. Temperature of growth was 30° C. Bound hydroxylamine was determined at the start of the experiment and, after the growth had started, at different intervals. Before the samples were taken the flasks were given a good shake. Samples of 1 ml were taken with sterile pipets from the growth solution and placed immediately into test tubes containing 0.5 ml 12 *N* H_2SO_4 and 0.5 ml sulphanilic acid (1 g sulphanilic acid in 100 ml of 30 % acetic acid). The tubes were kept for 6 h in a boiling water bath. Table 1 gives the data of two experiments performed according to this method.

Table 1.

Nitrogen nutrition	Period of growth, h	pH	Bound hydroxyl-amine (extinction)	Nitrogen nutrition	Period of growth, h	pH	Bound hydroxyl-amine (extinction)
	Experiment 1				Experiment 2		
N_2	0	7.2	0	N_2	0	7.2	0
	30	7.0	0		20	6.9	18
	42	6.5	10		40	6.4	26
40 mg % NH_4^+	0	7.2	0	40 mg % NH_4^+	0	7.2	0
	30	7.1	0		20	6.6	5
	42	6.2	5		40	6.4	26
40 mg % NO_3^-	0	7.2	0	40 mg % NO_3^-	0	7.2	0
	30	6.8	2		20	6.7	8
	42	6.4	63		40	6.5	49

Controls subtracted

In general, determination of hydroxylamine in the bacterial cultures was uncertain because the sugar-containing solution turns brownish during the hydrolysis.

The results show, however, that while the Azotobacter grows in the nutrient solution with a small inoculation when many hours are required before the cells have multiplied sufficiently to enable detection of nitrogen fixation and assimilation by ordinary methods of analysis, no clear difference due to the nature of nitrogen nutrition can be distinguished in the formation velocity of bound hydroxylamine. Yet after 40 h the amount of bound hydroxylamine was much greater in the cultures on nitrate nitrogen than in those on molecular or ammonium nitrogen.

Experiments of short duration by using heavy inoculation

Azotobacter was cultivated in 200 ml of nitrogen-free nutrient solution in 1 litre flat flasks. The flasks were placed on their broad sides in a cupboard the temperature of which was maintained by means of an electric heater at about 30° C during the day but fell during the night (in about 14 h) to about 18° C. The period of growth was 48 h. Bacteria were separated by centrifugation in the morning when after the low night temperature the amount of bound hydroxylamine in the cells was only about one third of that contained in the bacteria grown at an even temperature of 30° C. The bacteria isolated from 200 ml of culture were suspended in 600 ml of nitrogen-free nutrient solution, the suspension was shaken to as homogeneous a mass as possible and divided in 3 portions, 200 ml each, in 1 litre flat flasks. The flasks contained:

1. No combined nitrogen
2. $(NH_4)_2HPO_4$
3. KNO_3

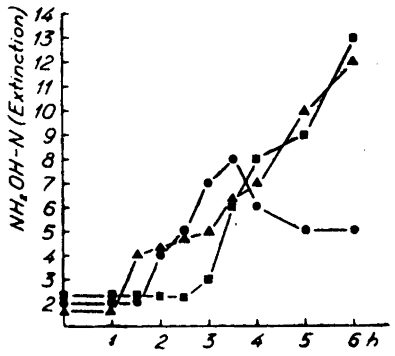

Fig. 1. Formation of bound hydroxylamine in parallel experiments with molecular nitrogen, nitrate nitrogen (40 mg %) and ammonium nitrogen (40 mg %).

●—● N_2
■—■ NH_4^+
▲—▲ NO_3^-

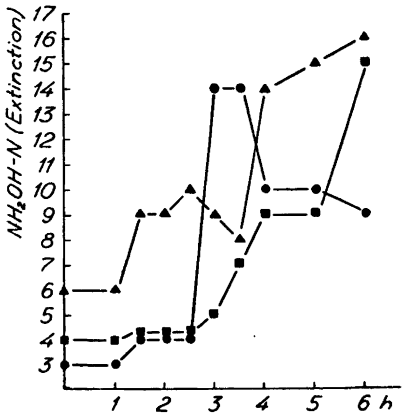

Fig. 2. Formation of bound hydroxylamine in parallel experiments with molecular nitrogen, nitrate nitrogen (40 mg %) and ammonium nitrogen (40 mg %).

●—● N_2
■—■ NH_4^+
▲—▲ NO_3^-

Total N of the bacterial mass after extraction with trichloracetic acid was as follows:

N-nutrition	N_2	NH_4	NO_3
Time min.	*Total N mg/ml*	*Total N mg/ml*	*Total N mg/ml*
0	*0.058*	*0.054*	*0.058*
60	*0.059*	*0.058*	
120		*0.067*	
180	*0.068*	*0.069*	*0.068*
210	*0.070*	*0.070*	
360	*0.070*	*0.070*	*0.069*

Temperature was 30° C. pH was in the beginning of each experiment 6.8. It fell during the experiment on N_2 and NH_4^+ nutrition to pH 6.6—6.5, on NO_3^- to 6.7. The amount of nitrogen added to different flasks are mentioned below in connection with the results.

From each flask a sample was taken with sterile pipet in the beginning of the experiment for determination of pH, hydroxylamine and total N. In the course of the experiment samples were taken in the same way at intervals of 30 mins. Total N of the bacterial mass was determined after extraction with trichloracetic acid (see below). A

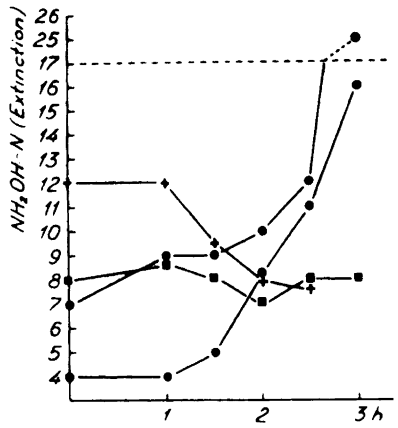

Fig. 3. Formation of bound hydroxylamine in parallel experiments with molecular nitrogen and ammonium nitrogen (2 mg % and 4 mg %).

●—● N_2
■—■ NH_4—N (2 mg)
+—+ NH_4—N (4 mg)

number of low-molecular substances had by then dissolved from the cells but the results are, however, comparable with each other to a great extent.

For determination of bound hydroxylamine, 5 ml of the sample were put in a centrifuge tube kept on ice. After centrifugation for 9 min the clear solution was poured away (in some experiments 1 ml of it was used for determination of bound hydroxylamine in the solution), 5 ml of ice-cold distilled water were added, shaken with the bacterial mass and re-centrifuged for 9 min. The washing water was poured off and replaced with 5 ml of 8 % trichloracetic acid. The bacterial mass was mixed well with the solution and the suspension was kept for 24 h in the ice-box. The procedure from taking the sample up to placing the bacterial mass with trichloracetic acid solution in the ice-box was carried out in 20—22 min. After 24 h, the suspension with trichloracetic acid was centrifuged for 15 min, a 1 ml sample was taken from the clear solution and bound hydroxylamine was determined in it in the manner described above.

Results of four experiments have been illustrated by curves in Figs. 1, 2, 3 and in Table 2.

Determination of bound hydroxylamine in nutrient solutions from which bacteria were separated by centrifugation, gave high extinction values right from the start of the experiment. The cause of this may be the heating of the sugar-containing solution at a strongly acid reaction as mentioned above. Determination of hydroxylamine is then uncertain. However, the values of hydroxylamine seem to rise in the nutrient solution somewhat parallel with the increase of the cell mass.

DISCUSSION

The results recorded above show that in the experiments of short duration, in which great amounts of bacterial mass were used for inoculation and therefore fixation of nitrogen and assimilation of ammonium nitrogen could be noted within 1—1 ½ h, bound hydroxylamine was formed more rapidly in the bacteria on nitrate and molecular nitrogen than on ammonium nitrogen. In the latter case bound hydroxylamine could not be noted until after 3 h, whereas

Table 2.

No	Nitrogen nutrition	Duration of growth min	pH	Ammonia N mg/ml	Bound hydroxylamine (extinction) in separated bacterial mass
1	N_2	0	6.8		0 (7)
		60			2 (9)
		90			2 (9)
		120			3 (10)
		150			5 (12)
		180	6.5		18 (25)
2	N_2	0	6.8		0 (4)
		60			0 (4)
		90			1 (5)
		120			4 (8)
		150			7 (11)
		180	6.5		12 (16)
3	NH_4^+—N, 2 mg %	0	6.8	0.019	0 (8)
		60		0.011	1 (9)
		90			0 (8)
		120		0.003	0 (7)
		150			0 (8)
		180	6.5		0 (8)
4	NH_4^+—N, 4 mg %	0	6.8		—
		60			0 (9)
		90			0 (8)
		120			0 (7)
		150	6.5		0 (8)

The numbers in parentheses indicate the readings.

in the former case it was regularly found after 1 ½—2 h. There is naturally the possibility that the ammonium content, which is always much higher in experiments with ammonium sulphate than when ammonium is possibly formed as an intermediate at the N_2-fixation, should have a retarding effect on the formation of bound hydroxylamine. However, in our experiments, in which the concentration of ammonium nitrogen varied from 2 to 40 mg % no formation of bound hydroxylamine could be noted even at the lowest concentration after 3 h. At that time ammonium nitrogen was completely used up. Our experimental material therefore speaks for the concept that in nitrogen fixation and nitrate reduction hydroxylamine is formed before the complete

reduction of nitrogen to ammonia. Accordingly, the results favour the intermediary nature of hydroxylamine.

On the other hand, it appears from the results that ammonium or amino nitrogen is oxidized to some extent. Otherwise it is not possible to explain the formation of bound hydroxylamine after 3 h in nutrient solution with 40 mg % ammonium nitrogen since after 6 h about 90 % of ammonium nitrogen were still left in the solution. Since the increase of bound hydroxylamine does not begin until the assimilation of nitrogen has well started it is possible that either amide or amino group is oxidized. However, no definite conclusions can be drawn from this.

SUMMARY

In cultures of *Azotobacter vinelandii* in which heavy inoculation was used and accordingly, nitrogen fixation and assimilation of nitrogen compounds were detectable by ordinary chemical analyses within 1—2 h, bound hydroxylamine could be found in 1 ½—2 h when molecular nitrogen and nitrate nitrogen served as the source of nitrogen. When ammonium nitrogen provided the nitrogen nutrition, bound hydroxylamine was not found during the first 3 h, later it appeared in increasing amounts.

The results are considered to support the concept that hydroxylamine is formed in nitrate reduction and in nitrogen fixation as an intermediate before ammonia. On the other hand, oxidation of ammonia to hydroxylamine also appears from the results obtained.

REFERENCES

1. Blom, J. *Ber.* **59** (1926) 121.
2. Endres, G. *Ann.* **518** (1935) 109.
3. Speck, J. F. *J. Biol.Chem.* **168** (1947) 403.
4. Elliot, W. H. *Biochem. J.* **42** (1948) V.
5. Waelsch, H., Borek, E., and Grossowicz, N., *Abstracts, Am. Chem. Soc.*, 116th meeting 54 C (1949); *Federation Proc.* **9** (1950) 242.
6. Grossowicz, N., Wainfan, E., Borek, E., and Waelsch, H. *J. Biol. Chem.* **187** (1950) 111.
7. Stumpf, P. K., and Loomis, W. D. *Arch. Biochem.* **25** (1950) 451.
8. Virtanen, A. I., Hakala, M., and Järvinen, H. *Suomen Kemistilehti* B, **22** (1949) 23.
9. cf. Virtanen, A. I. *Ann. Acad. Sci. Fennicae, Ser. A.* II. Chem. (1950) no. 39.
10. Csáky, T. Z. *Acta Chem. Scand.* **2** (1948) 450.
11. Miller, G. L., and Miller, E. E. *Anal. Chem.* **20** (1948) 481.
12. Virtanen, A. I. *Z. physiol. Chem.* **134** (1924) 300.

Received November 9, 1950.