1. Christiansen, J. A. *Acta Chem. Scand.* **3** (1949) 493.
2. Christiansen, J. A. *Advances in Catalysis* **5** (1953) 311.
3. Loewenstein, O. and Sand, A. *J. Exptl. Biol.* **13** (1936) 416.
4. Sand, A. *Proc. Roy. Soc. London* **B 125** (1938) 524.
5. Loewenstein, O. and Roberts, T. D. M. *J. Physiol. London* **110** (1949) 392.

Received September 17, 1957.

On Hydroxylamine Compounds in *Azotobacter* Cultures. I. Formation of Hydroxylamine Compounds

NILS-ERIK SARIS and ARTTURI I. VIRTANEN

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

Blom [1] was the first to demonstrate small amounts of hydroxylamine in nitrogen-fixing *Azotobacter* cultures, and later on bound hydroxylamine was found by Endres [2]. Hydroxylamine was formed only from nitrate or molecular nitrogen but not from ammonium nitrogen. On the other hand Virtanen and Hakala [3] observed that about the same amount of bound hydroxylamine was found in the nutrient solutions of *Azotobacter vinelandii* both when nitrate nitrogen, ammonium nitrogen, and molecular nitrogen were the sources of nitrogen. Virtanen and Järvinen [4] investigated the formation of bound hydroxylamine in the cell suspension of *Azotobacter vinelandii* and found that it was formed both from N_2-, NO_3-, and NH_4-N, although more rapidly from molecular nitrogen and nitrate nitrogen than from ammonium nitrogen. These authors determined bound hydroxylamine in the cell mass separated by centrifugation.

The present authors have now further studied the formation of hydroxylamine compounds found in the nutrient solution of *Azotobacter*. Under the experimental conditions used the enrichment of hydroxylamine compounds in cells was too scant to make a closer investigation of these compounds possible. On the other hand bound hydroxylamine was enriched in the nutrient solution in such amounts that a closer investigation of them could be attempted.

Nutrient solution. 1.5 g Burk's salt, 10 mg $Fe_2(SO_4)_3$, 15 mg $FeSO_4$, $7H_2O$, 0.25 mg Na_2MoO_4 per litre, pH 7.2. In the experiments proper glycerol was the source of carbon (*cf.* below); the source of nitrogen is mentioned in connection with the experimental results.

Determination of bound hydroxylamine. Csáky's method was used, with the modification that the hydrolysis time was shortened to one hour. To destroy nitrite a pinch of urea was added. Determinations were made both from 2 and 10 ml.

Azotobacter strain. In preliminary experiments the largest amount of bound hydroxylamine was found in cultures of *Azotobacter chroococcum* K 1. In cultures of other investigated strains (*A. vinelandii* Kluyver, *vinogradsky* Kluyver, *beijerinckii*, and a not closer characterized strain isolated from soil) a much smaller amount of bound hydroxylamine, if any, was enriched.

Effect of the carbon source. Because Endres obtained the largest amount of bound hydroxylamine when lactate was the source of carbon, growth experiments were performed with different carbon compounds (glycerol, Ca-lactate, saccharose, ethanol, acetone, sorbitol). In the concentrations investigated glycerol was as good a carbon source as lactate, and it was used in the experiments proper. The large amounts of hydroxylamine found by Endres in lactate nutrient solutions were not, however, even approximately obtained. He found maximum 2×10^{-4} mole NH_2OH per 10 ml, the present authors about 10^{-5} only. This circumstance made the isolation of hydroxylamine compounds and their characterization difficult.

Cultivation methods. Method 1. Azotobacter was allowed to grow without shaking in 1 litre flat flasks containing 200 ml of nutrient solution each. 200 ml of nutrient solution without combined nitrogen was inoculated with 2.5 ml of a 4-day-old *Azotobacter* culture. The experiment was terminated after 3 days, the maximum amount of bound hydroxylamine being then found in the solution. The influence of the age of the cultures on the content of bound hydroxylamine in the nutrient solution can be seen from the following results with *Azotobacter chroococcum* K 1:

Time after inoculation, days	1	2	3	4	5	6
Cells formed, mg/10 ml	0	30	120	165	190	205
NH_2OH after hydrolysis, μg N/10 ml	0	0.54	1.40	0.95	0.30	0

The effect of the nitrogen source on the enrichment of hydroxylamine compounds in the nutrient solution was determined after 3 days of growth in glycerol nutrient solutions in which KNO_3, $(NH_4)_2CO_3$, urea, or N_2 (without combined nitrogen) were the sources of nitrogen. The amount of each nitrogen compound used was 60 mg N per 200 ml nutrient solution. The results can be seen in Table 1.

Table 1. Effect of the nitrogen source on the enrichment of bound hydroxylamine in the nutrient solution of *A. chroococcum.*

Nitrogen nutrition	NH_2OH-N μg/10 ml	Cells mg/10 ml	Assimilated N mg/10 ml	Excret. total N mg/10 ml
N_2	1.46	160	2.15	0.08
KNO_3	1.04	85	1.23	—
$(NH_4)_2CO_3$	1.80	40	0.52	—
$CO(NH_2)_2$	1.62	140	2.05	—

Method 2. 1 Litre Kluyver flasks contained 200 ml of the above-mentioned nutrient solution. A vigorous current of air was passed through the solution after addition of bacterial mass. Under these conditions hydroxylamine was accumulated in the solution only when a large amount of bacteria was suspended in the nutrient solution.

The bacterial mass used was separated by centrifugation from two-days-old liquid cultures and suspended into the nutrient solution using a Waring-Blendor homogenisator. 250 mg of *Azotobacter* mass were added to each nutrient solution. KNO_3 (60 mg N/200 ml), urea (60 mg N/200 ml), and N_2 (no combined nitrogen in the nutrient solution) were the sources of nitrogen. The results can be seen in Fig. 1.

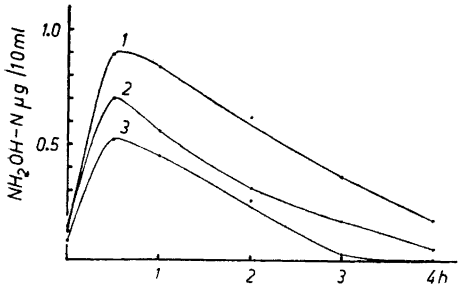

Fig. 1. Bound hydroxylamine in a vigorously aerated suspension of *A. chroococcum* in nutrient solutions with potassium nitrate (1), molecular nitrogen (2), and urea (3) as nitrogen source.

The results recorded above show that bound hydroxylamine, accumulated in the nutrient solution, disappears from flask cultures after the 4th day and from aerated suspensions of bacteria within a few hours. This disappearance may depend on assimilation or decomposition of the substance in the nutrient solution. It was also found that the yield of hydroxylamine decreases when the pH falls below 7.3. Hydroxylamine compounds were not demonstrable in cultures with a pH below 6.8.

In order to find out to what extent bound hydroxylamine, accumulated in the nutrient solution, disappears in a vigorously aerated suspension at different pH values, a fraction containing bound hydroxylamine, separated with Amberlite IR-120, was added to two suspensions of *A. chroococcum* K 1 in the nutrient solution. The pH of the one solution was regulated to 7.3 and that of the other to 6.5. An equally strong current of air was

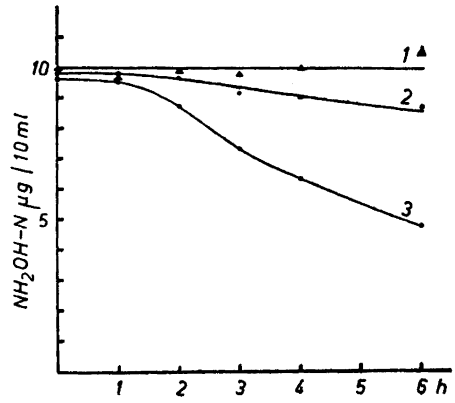

Fig. 2. Disappearance of added hydroxylamine fraction without *Azotobacter* (1), with *Azotobacter* suspension, pH 6.5 (2), and with *Azotobacter* suspension, pH 7.3 (3).

passed through both suspensions in a Kluyver flask. In the control the nutrient solution containing the hydroxylamine fraction but no bacteria was aerated. The results can be seen in Fig. 2.

These results indicate that the hydroxylamine fraction is stable without *Azotobacter* during the experimental period, *i. e.* for 6 h, and that in experiments with *Azotobacter* bound hydroxylamine disappears much faster at pH 7.3 than at pH 6.5. The results suggest accordingly that the absence of bound hydroxylamine in *Azotobacter* cultures at pH 6.5 is due to a reduced formation of hydroxylamine compounds.

Since hydroxylamine compounds are formed in our *Azotobacter* cultures from NH_4—N as rapidly as from N_2, and identical compounds seem to be in question (*cf.* paper II), there is no evidence any longer of an oxidative step in aerobic N_2 fixation. On the basis of their results with *Clostridium* Virtanen and Hakala [5] years ago suggested that N_2 fixation by anaerobic organisms is a pure reduction, di-imide being the first intermediate, and the same mechanism can now be suggested also for aerobic N_2 fixation.

1. Blom, J. *Zentr. Bakteriol. Parasitenk. II* **84** (1931) 60.
2. Endres, G. *Ann.* **518** (1935) 109.
3. Virtanen, A. I. and Hakala, M. *Unpublished.*
4. Virtanen, A. I. and Järvinen, H. *Acta Chem. Scand.* **5** (1951) 220.
5. Virtanen, A. I. and Hakala, M. *Suomen Kemistilehti B* **22** (1949) 23; *Acta Chem. Scand.* **3** (1949) 1044.

Received September 22, 1957.

On Hydroxylamine Compounds in *Azotobacter* Cultures. II. On the Chemical Nature of the Bound Hydroxylamine Fraction in *Azotobacter* Cultures

NILS-ERIK SARIS and ARTTURI I. VIRTANEN

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

In the previous communication [1] it was reported that bound hydroxylamine was accumulated in the nutrient solution of *Azotobacter*. Attempts to isolate and characterize these hydroxylamine compounds are reported in this paper.

Behaviour on ion exchange columns. Bound hydroxylamine from the nutrient solution passed through a slightly basic resin (Amberlite IR-4B), but was absorbed by a strongly basic one (IR-400). It was eluted quantitatively with diluted acetic or formic acid from the latter. On the other hand it was also absorbed almost quantitatively by acid resins from more concentrated solutions. Only some per cent of the total bound hydroxylamine passed through the Amberlite IR-120 column. From this column the amino acids and part of the hydroxylamine compounds could be eluted by 1 N ammonia, the latter compounds quantitatively only with 10 % ammonia. Paper chromatographic characteristics showed that the bound hydroxylamine eluted together with amino acids by dilute ammonia was similar to that eluted by more concentrated ammonia.

Separation of the bound hydroxylamine absorbed by the Amberlite IR-120 column. The fraction obtained from the glycerol cultures (N_2 as source of nitrogen) of *Azotobacter* was investigated by paper chromatography. The fraction was obtained by evaporating the culture solution to a small volume *in vacuo*, precipitating mucous substances with ethanol, evaporating ethanol *in vacuo* from the clear solution, passing the solution through the Amberlite IR-120 column, eluting the column

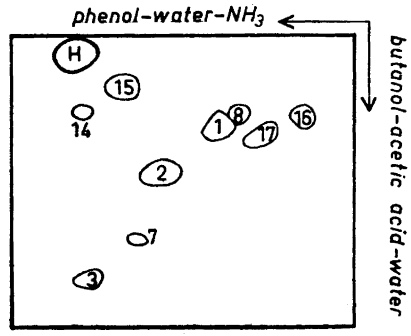

Fig. 1. Two-dimensional run of amino acids and bound hydroxylamine (formed in the culture solution of *A. chroococcum* K 1 grown in glycerol-flask-cultures for 3 days) after absorption in Amberlite IR-120 resin and elution with 1 N ammonia. H = bound hydroxylamine (a weak greyish spot with ninhydrin), 1 = gly, 2 = ala, 3 = val, 7 = tyr, 8 = ser, 14 = arg, 15 = lys, 16 = asp, 17 = glu.