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Heterotrophic Nitrification by *Arthrobacter* sp.

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Arthrobacter sp. isolated from sewage oxidized ammonium to hydroxylamine, a bound hydroxylamine compound, a hydroxamic acid, a substance presumed to be a primary nitro compound, nitrite, and nitrate. The concentration of free hydroxylamine-nitrogen reached 15 μg/ml. The identification of hydroxylamine was verified by mass spectrometric analysis of its benzophenone oxime derivative. The bound hydroxylamine was tentatively identified as 1-nitrosoethanol on the basis of its mass spectrum, chemical reactions, and infrared and ultraviolet spectra. Hydroxylamine formation by growing cells was relatively independent of *p*H, but the accumulation of nitrite was strongly favored in alkaline solutions. The formation of hydroxylamine but not nitrite was regulated by the carbon to nitrogen ratio of the medium. The hydroxamic acid was the dominant product of nitrification in iron-deficient media, but hydroxylamine, nitrite, and 1-nitrosoethanol formation was favored in iron-rich solutions. Heterotrophic nitrification by *Arthrobacter* sp. was not inhibited by several compounds at concentrations which totally inhibited autotrophic nitrification.

Nitrification is the biological conversion of nitrogen in either inorganic or organic compounds from a reduced to a more oxidized state (1). It is now evident that a variety of heterotrophs oxidize nitrogen compounds (8, 11). Not only do these organisms oxidize ammonium, hydroxylamine, and nitrite but also a number of organic nitrogenous substances. Among the identified products of heterotrophic nitrification are hydroxamic acids (20), oximes (26), amine oxides (3), nitroso compounds (19), nitro compounds (16), nitrite, and nitrate (5). Nevertheless, neither the biochemical mechanisms nor the ecological significance of most of these reactions are known.

This study was initiated to understand more fully the biochemistry and importance in nature of heterotrophic nitrification. The present report describes the products of heterotrophic nitrogen oxidation and the pattern of their formation by a strain of *Arthrobacter*. Results of the biochemical investigations and a demonstration of the functioning of heterotrophic nitrification in samples from natural ecosystems will be published separately.

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MATERIALS AND METHODS

To isolate a nitrifying heterotroph, an enrichment culture was set up with 0.2% acetamide as the carbon source in an inorganic medium containing 8.2 g of KH_2PO_4, 1.6 g of NaOH, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, and 0.5 mg each of $CaSO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, and 0.5 mg of $ZnSO_4 \cdot H_2O$ per liter of distilled water. The medium was adjusted to *p*H 7.0, inoculated with municipal sewage, and incubated on a rotary shaker (120 rev/min) at 28 C for 1 week. Because active formation of nitrite was observed, the responsible microorganism was isolated by streaking the enrichment on the same medium solidified by the addition of 1.7% agar. Separate colonies were picked and individually tested for nitrifying activity by growing them axenically in the liquid.

One isolate was particularly active. Since this strain nitrified even better when grown with 1.69% sodium acetate as carbon source and 0.47% $(NH_4)_2SO_4$ as nitrogen source than when given acetamide, these sources of carbon and nitrogen were used during subsequent studies. When this organism, a bacterium, was grown in nutrient broth at 28 C, it was found to change morphology during its life cycle. One day after inoculating nutrient broth, gram-negative to gram-variable rods, 0.5 to 1.0 μm wide and 2.0 to 5.0 μm long, were observed. By day 2 many chains of rods were noted. Most chains had disappeared and gram-negative rods together with gram-variable coccoidal forms were predominant on

day 3. By day 4, gram-negative rods and gram-negative to gram-variable coccoidal forms were evident.

The bacterium was further characterized by the procedures described by Skerman (23). It gave negative tests for acetylmethylcarbinol formation, nitrate reduction, and starch hydrolysis. It formed alkali but no gas from arabinose, xylose, glucose, fructose, lactose, and sucrose. It was catalase-positive, grew on citrate and $(NH_4)_2SO_4$, produced H_2S, and was motile in young cultures. This bacterium, identified as a strain of *Arthrobacter*, resembles no species described to date.

Arthrobacter sp. was grown in the inorganic medium described above amended with 16.9 g of sodium acetate and 4.7 g of $(NH_4)_2SO_4$. The concentrations of acetate and ammonium corresponded to 3.0 mg of carbon and 1.0 mg of nitrogen per ml. The cultures were grown at 28 C on a rotary shaker operating at 120 rev/min, unless otherwise indicated.

Protein was determined by the method of Lowry et al. (14) with crystalline bovine serum albumin as the standard. The soluble protein content of the culture supernatant fluid was determined after removing the cells by centrifugation at 10,000 × *g* for 20 min. The method of Magee and Burris (15) was used to estimate free hydroxylamine, and nitrite was determined by the procedure of Montgomery and Dymock (18). The phenoldisulfonic method (L.G. Morrill, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1959) was used to measure nitrate, and hydroxamic acids were estimated by the technique of Lipmann and Tuttle (13) with acetohydroxamic acid as standard. Primary aliphatic nitro compounds were determined by the method of Matsumoto et al. (17); free and bound hydroxylamine did not interfere in the analysis.

To determine bound hydroxylamine, that is, a compound which liberates free hydroxylamine when boiled in mildly acidic conditions, 1.0 ml of a sulfanilic acid-sulfate solution (0.346% sulfanilic acid and 2.72% $KHSO_4$ in distilled water) was added to 1.0 ml of a sample containing no more than 1.5 μg of bound hydroxylamine-nitrogen per ml. This mixture was boiled for 5 min and cooled, and the free hydroxylamine liberated was estimated. Nitrite interfered in the determination, but any nitrite present was destroyed by adding 0.5 ml of a 0.5% aqueous sulfamic acid solution to 0.5 ml of a sample containing not more than 100 μg of nitrite-nitrogen per ml. After 10 min, by which time all the nitrite had been destroyed, the sample was assayed for bound hydroxylamine. The sulfamic acid neither destroys nor interferes in the determination of the bound hydroxylamine formed by *Arthrobacter* sp.

Gas chromatographic analysis of the bound hydroxylamine compound was performed with a stainless-steel column (36 by 0.24 cm) packed with Porapak Q, 100 to 200 mesh. The column was mounted in a gas chromatograph, Varian Aerograph series 1700, equipped with a flame ionization detector. The flow rate of the carrier gas, N_2, was 50 ml/min. The temperatures of the injector, column, and detector were 140, 120, and 205 C, respectively. To obtain a mass spectrum of the compound, the Porapak Q column was mounted into a combined gas chromatograph-mass spectrometer (model 270, Perkin Elmer, Norwalk, Conn.). The sample was injected on the column, separated in the chromatograph unit, and then subjected directly to an ionization potential of 50 ev at ambient temperature in the mass spectrometer.

Nitroso compounds were determined by the photochemical method of Daiber and Preussmann (4), in which the nitroso group is cleaved and quantitatively oxidized to nitrite under strong ultraviolet (UV) light. Nitrite was then estimated by the method of Montgomery and Dymock (18). Nitrous oxide, nitric oxide, and nitrogen dioxide were determined by the procedures of C.W. Blanckenberg (M.S. thesis, Cornell University, Ithaca, N.Y., 1969).

To prepare benzophenone oxime from the biologically formed hydroxylamine, 0.10 g of benzophenone and 4.0 g of KOH were added to 200 ml of the culture supernatant fluid. This solution was heated at 80 C for 45 min, cooled, acidified to *p*H 3.0 with 1.0 N sulfuric acid, and extracted with ethyl ether. The extract was dried over anhydrous Na_2SO_4 and concentrated in a flash evaporator. The benzophenone oxime and excess benzophenone in the concentrated ether extract were separated by thin-layer chromatography by using silica gel HF-254+366 as support and benzene as solvent. The oxime was extracted from the silica gel with ethyl ether, and the ether was evaporated to yield the purified hydroxylamine. To obtain a mass spectrum of the oximes, a solid sample was inserted into the spectrometer by a direct inlet device and subjected to an ionization potential of 70 ev at ambient temperature.

To obtain infrared spectra, the metabolite was extracted from the aqueous solution with ethyl ether. The ether extract was dried over anhydrous Na_2SO_4 and evaporated at 30 C under vacuum. The compound was then dissolved in chloroform, and an infrared spectrum of this solution was taken using a Beckman IR10 double-beam, scanning, infrared spectrophotometer. The sample was contained in an infrared liquid cell having windows made of NaCl.

To verify the proposed structure of the bound hydroxylamine, which was tentatively identified as 1-nitrosoethanol, this compound was synthesized. Since no published method for the synthesis exists, a procedure was devised based on several known reactions. Because alkyl halides react with nitrous acid to form a mixture of nitroso alkanes, alkyl nitrates, and alkyl nitrites (24), it was postulated that the reaction of acetyl chloride with silver nitrite might give rise to CH_3CO-NO as well as other products. Reduction of the carbonyl group of this nitroso compound with sodium borohydride should yield 1-nitrosoethanol.

The synthesis was conducted by placing 1.0 g of $AgNO_2$, 50 ml of dry ethyl ether, and 0.5 ml of acetyl chloride in a glass-stoppered 500-ml Erlenmeyer flask, and the contents of the stoppered container were mixed for 15 min. The solution was passed through Whatman no. 1 filter paper, and the filtrate was concentrated by evaporation at 30 C under vacuum. When about 5 to 10 ml of ether remained, a phosphate-borohydride solution (0.5 g of $NaBH_4$ in 25 ml of 0.2 M K_2HPO_4) was added

quickly and promptly, and the mixture was stirred for 20 min. One-third of this solution was distilled under vacuum at 40 C. This distillate was found to contain a bound hydroxylamine compound, presumably 1-nitrosoethanol, at a concentration of about 20 μg of nitrogen per ml.

Benzophenone was obtained from Aldrich Chemical Co. (Milwaukee, Wisc.), silica gel HF-254+366 from Brinkmann Instruments, Inc. (Westbury, N.Y.), Porapak Q from Waters Associates, Inc. (Framingham, Mass.), and ethyl nitrate and *n*-butyl nitrite from Pfaltz & Bauer Inc. (Flushing, N.Y.). A sample of 2-chloro-6-(trichloromethyl)pyridine was obtained from Dow Chemical Co. (Midland, Mich.).

RESULTS

When grown in a medium containing $(NH_4)_2SO_4$, acetate, and inorganic salts, *Arthrobacter* sp. excreted hydroxylamine, a bound hydroxylamine compound, a hydroxamic acid, a substance presumed to be a primary nitro compound, nitrite, and nitrate. The sequence of appearance of some of the products of nitrification in relation to bacterial growth is shown in Fig. 1. The concentration of hydroxylamine increased during the exponential growth phase and then declined. Concomitant with the disappearance of hydroxylamine, the appearance of bound hydroxylamine, the primary nitro compound, nitrite, and nitrate in the supernatant fraction became evident. The primary nitro compound accumulated to a concentration of 3.0 μg of nitrogen per ml. In addition (not shown), the apparent hydroxamic acid appeared in the supernatant liquid at the same time or just after the hydroxylamine, and it accumulated to a maximum concentration of 6.0 μg of nitrogen per ml and then declined gradually to about 4.0 μg/ml. Nitrous oxide, nitric oxide, and nitrogen dioxide were not detected in the gas phase above the growing culture. As the organism multiplied, the *p*H rose from 7.0 to 9.2, and large amounts of soluble protein were released into the supernatant fluid, the accumulation of which paralleled growth and amounted to a maximum of 720 μg/ml.

To verify that the substance giving a positive reaction in the Magee and Burris (15) test was indeed hydroxylamine, the supernatant fluid of a culture giving a positive reaction was concentrated 20-fold by evaporation under vacuum. Paper chromatography of this concentrated solution performed by the method of Bremner (2) revealed the presence of a yellow spot with an R_F value of 0.55. Authentic hydroxylamine gave a spot with exactly the same color and R_F value. The identity of hydroxylamine was further verified by converting the

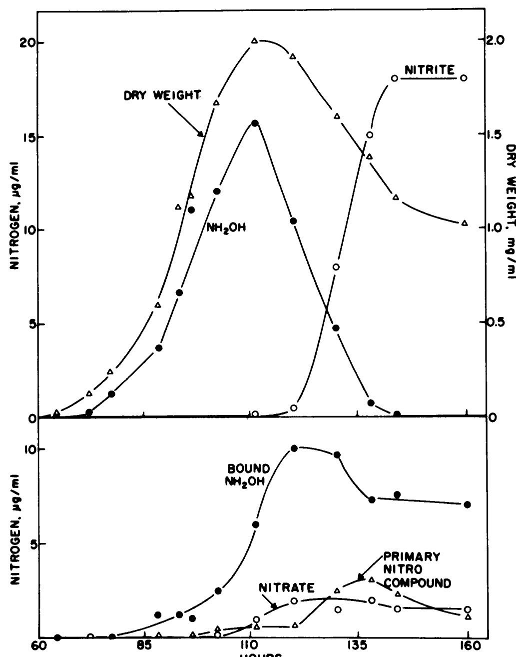

FIG. 1. *Formation of several nitrogenous compounds during the growth of Arthrobacter sp.*

product to benzophenone oxime. The oxime prepared from the biologically formed hydroxylamine had exactly the same R_F value, 0.35, as the oxime prepared from authentic hydroxylamine when chromatographed on silica gel HF-254+366 with benzene as solvent. Moreover, the mass spectra of the two oximes were identical.

To establish the identity of the bound hydroxylamine, the supernatant fluid of a 5-day-old culture was acidified to *p*H 6.5, and about one-fifth of this sample was distilled in a vacuum evaporator at 40 C and collected. The bound hydroxylamine partly codistilled with the water and was concentrated in the distillate. When the distillate was examined by gas chromatography, the chromatogram contained one major peak with a retention time of 180 sec as well as several minor peaks. The 180-sec peak appeared to represent the bound hydroxylamine because it was not present in gas chromatograms of distillate in which the bound hydroxylamine had been destroyed by boiling under weakly acidic conditions (that is, in the bound hydroxylamine test). The bound hydroxylamine did not decompose at room temperature when the aqueous solution was acidified to *p*H 1.0. The distillate contained no free hydroxylamine, nitrite, or primary nitro compounds, but boiling of the distillate for 10

min in the presence of 2.0 N alkali gave rise to traces of nitrite.

Nitrite was formed when an aqueous solution containing the bound hydroxylamine compound was subjected to UV light by the procedure of Daiber and Preussmann (4), and the amount of nitrite-nitrogen produced by the photolytic treatment equalled the amount of hydroxylamine-nitrogen obtained by boiling the compound in weak acid employed in the bound hydroxylamine test. Alkyl nitrites and nitrates and nitro and nitroso compounds are reportedly cleaved quantitatively under UV irradiation to form nitrite; however, alkyl nitrites are hydrolyzed rapidly in neutral and acidic aqueous solution (4, 24). Hence, because the bound hydroxylamine compound is stable in neutral and acidic aqueous solution, it is probably not an alkyl nitrite. By using several model compounds we found that organic nitrates and nitro compounds do not form hydroxylamine when boiled in the weak acid used in the bound hydroxylamine test. On the other hand, nitroso compounds presumably form hydroxylamine quantitatively when boiled in weak acid, probably because nitroso compounds isomerize to the corresponding oximes when heated in acid, and the oximes thus formed are rapidly hydrolyzed in the hot acid to hydroxylamine (24).

To identify the carbon moiety of the presumed nitroso compound giving a test for bound hydroxylamine, its mass spectrum was obtained by means of a combined gas chromatograph-mass spectrometer. Only the carbon moiety is usually observed in the mass spectrum of nitroso compounds (9). The mass spectrum of the bound hydroxylamine contained a number of low-molecular-weight fragments (Fig. 2). The peaks at *m/e* 15, 27, 29, 31, and 45 suggest that the carbon moiety has the structure: CH_3CHOH. The fragments in the mass spectrum might then represent CH_3, CH_3C, CH_3CH_2, CH_2OH, CH_3CO, and CH_3CHOH for the peaks at *m/e* 15, 27, 29, 31, 43, and 45, respectively. The peaks at *m/e* 30 and 46 correspond to NO and NO_2 ions. These results indicate that the unknown is 1-nitrosoethanol.

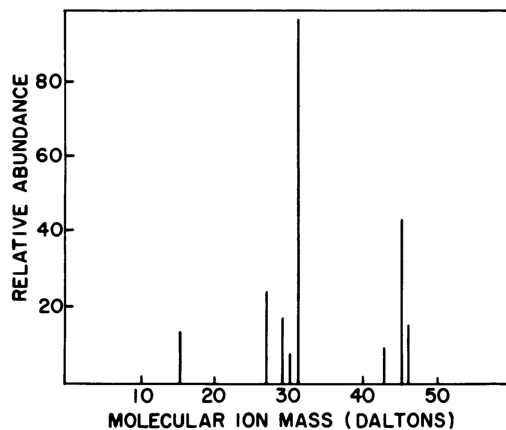

FIG. 2. *Mass spectrum of the bound hydroxylamine compound.*

Further evidence in support of the proposed structure was obtained by examining the UV absorption characteristics of synthetic 1-nitrosoethanol. An aqueous solution of this compound absorbed UV light moderately at 280 nm and showed strong absorption at 220 nm. The molar absorbancy at 280 nm was 260 cm. This corresponds with the molar absorption of nitroso monomers at that wavelength (22).

Neither the biological nor the chemical synthesis yielded sufficient quantities of 1-nitrosoethanol to allow for a well-resolved infrared spectrum of the purified compound. An infrared spectrum of the partly purified synthetic compound showed a broad OH-band at 3,400 cm^{-1}, a CH-band at 2,900 cm^{-1}, two sharp, strong NO-bands at 1,370 and 1,344 cm^{-1}, and broad CH-adsorption at 1,100-1,000 cm^{-1}. Although the spectrum of the partially purified synthetic product was not well resolved nor free of bands from contaminating materials, it was consistent with the proposed identification as 1-nitrosoethanol. The two sharp bands at 1,370 and 1,344 cm^{-1} indicate that 1-nitrosoethanol is present in chloroform solution as a *cis*-dimer (9).

On the basis of the colorimetric procedure of Matsumoto et al. (17), it appears that small amounts of a nitro compound were excreted by the bacterium. The colorimetric procedure detects primary nitro compounds with a reasonable degree of specificity, and the metabolite may indeed be a primary nitro compound. However, inasmuch as the time of appearance of the compound did not suggest that it was a precursor of the other products, no further characterization was attempted.

While the bacterium was actively proliferating, a compound accumulated in the supernatant fluid which reacted with ferric iron used in the hydroxamic acid test (13) to form a reddish-brown complex. The compound was stable in aqueous solution at *p*H 1.0. To purify it, the supernatant fluid was acidified to *p*H 2.0 with HCl and extracted with *n*-butanol. The butanol extract was in turn extracted with 0.5 N aqueous KOH, the unknown migrating into the alkaline aqueous phase. The aqueous phase was then removed and neutralized with dilute acid. The compound was purified by thin-layer

chromatography. On silica gel HF-254+366 with *n*-butanol-acetic acid-water (4:1:5) as solvent system, the substance had an R_F of 0.70. Hydrolysis of the chromatographically purified material in 6 N H_2SO_4 for 30 min at 120 C yielded free hydroxylamine. After the hydrolysis, the compound gave a negative reaction with the ferric-iron reagent (13). These results are consistent with the hypothesis that the compound is a hydroxamic acid. The apparent lack of appreciable metabolism of the hydroxamic acid suggests that it is not an intermediate in the pathway of nitrification by the organism.

For reasons still unclear, the *p*H range for heterotrophic nitrification is frequently narrower than the range for growth of the organism (11). A study was performed to establish how the formation of hydroxylamine and nitrite was affected by *p*H. This inquiry assumes added importance because of observations that these two highly toxic products are generated heterotrophically in samples from natural ecosystems in a process which is markedly sensitive to acidity (Verstraete and Alexander, *unpublished data*). Phosphate (0.1 M) was used to buffer the medium at *p*H 6.0 to 8.0, and carbonate (0.1 M) for *p*H values of 9.0 and 10.0. At regular intervals during growth of the organism, the medium was adjusted to its original *p*H value. No growth was observed at *p*H 6.0 and 10.0 in a 7-day incubation period. The maximum amounts of hydroxylamine and nitrite formed at the intermediary *p*H values in this time period are presented in Table 1. The products of nitrification accumulated in smaller amounts in these buffered media than in solutions in which the *p*H was allowed to rise during the incubation. Because the maximum accumulation of nitrite occurred after the onset of autolysis (Fig. 1), the activity coefficient for nitrite was calculated using the maximum cell dry weight noted during the entire growth cycle. The calculations in Table 1 show that the yield of hydroxylamine per unit of cell weight is independent of *p*H, whereas the quantity of nitrite relative to cell mass was strongly favored in alkaline solutions.

To determine the influence on nitrification of the C/N ratio of components in the medium, a factor generally markedly affecting the magnitude of heterotrophic nitrification, the formation of hydroxylamine and nitrite was determined in media with C/N ratios ranging from 10:1 to 1:10 in cultures which were incubated for 7 days. The activity coefficients for hydroxylamine and nitrite were calculated in the same way as before. The results summarized in Table 2 show that, though the amounts of hydroxylamine did not vary considerably with the different C/N ratios, its formation per unit of cell weight was significantly higher at the lower C/N ratios. On the other hand, the yield of nitrite relative to bacterial mass was greatest at the higher C/N ratios. Particularly striking is the fact that the quantity of hydroxylamine was little influenced by the amount of carbon provided to the bacteria, in contrast with the quantity of nitrite, which was roughly proportional to the amount of acetate added initially.

The influence of iron concentration on nitrification was investigated because the microbial formation of hydroxamic acids is frequently related to the available iron content of the medium (20). The cultures were grown for 5 days in media with different concentrations of $FeCl_3$, and the solutions were periodically analyzed. The maximum amounts of four of the metabolites found during the period of growth are given in Table 3. The results demonstrate that the accumulation of three of the compounds was markedly enhanced by trace amounts of iron. The data also show that accumulation of the hydroxamic acid was inversely related to the iron concentration in the medium.

A variety of chemicals are potent inhibitors of autotrophic nitrification. Because such compounds might be useful in allowing for a differentiation between autotrophic and heter-

TABLE 1. *Influence of pH on growth and hydroxylamine and nitrite formation by Arthrobacter sp.*

*p*H	Maximum NH_2OH[a]	Growth at maximum NH_2OH[b]	μg of NH_2OH-N/mg dry wt	Maximum nitrite level[a]	Maximum growth[b,c]	μg of nitrite-N/mg dry wt[c]
7.0	2.10	0.08	26	0.15	1.00	0.15
7.5	5.00	0.22	22	0.65	1.60	0.41
8.0	4.50	0.18	25	1.1	1.64	0.67
9.0	2.95	0.13	22	3.3	0.62	5.3

[a] Expressed as micrograms of N per milliliter.
[b] Expressed as milligrams (dry weight) per milliliter.
[c] Maximum cell yield.

TABLE 2. *Influence of the C/N ratio of the medium on growth and nitrification by Arthrobacter sp.*

C/N ratio	Acetate-C[a]	Ammonium-N[a]	Maximum NH_2OH level[b]	Growth at maximum NH_2OH[c]	μg of NH_2OH-N/ mg dry wt	pH[d]	Maximum nitrite level[b]	Maximum growth[c]	μg of nitrite-N/ mg dry wt	pH[e]
10	3,000	300	14.4	0.60	24	8.3	8.6	0.97	8.9	9.4
3	3,000	1,000	16.5	0.75	22	8.5	14.	0.97	14.	9.3
1	1,000	1,000	18.0	0.56	32	8.5	3.6	0.57	6.3	8.6
0.3	330	1,000	12.0	0.20	60	8.6	1.4	0.22	6.4	8.6
0.1	100	1,000	9.7	0.06	162	7.3	0.4	0.06	7.	7.3

[a] Expressed as micrograms per milliliter.
[b] Expressed as micrograms of N per milliliter.
[c] Expressed as milligrams (dry weight) per milliliter.
[d] At time of maximum hydroxylamine accumulation.
[e] At time of maximum nitrite accumulation.

TABLE 3. *Influence of iron concentration on nitrification by Arthrobacter sp.*

Ferric iron added (μg/ml)	Maximum observed (μg of nitrogen/ml)			
	Hydroxylamine	Nitrite	1-Nitrosoethanol	Hydroxamic acid
0.0	0.70	0.1	1.0	7.0
0.1	20.0	5.0	6.7	3.0
1.0	22.4	1.7	8.9	0.5
10.0	25.1	2.0	10.	0.5

otrophic nitrogen oxidation in natural environments, the influence of certain of these chemicals on *Arthrobacter* sp. was tested. The compounds were sterilized by filtration and added to the medium just before inoculation. Growth and the accumulation of hydroxylamine and nitrite during a 7-day incubation period were not altered by 10^{-2} M 1-allyl-2-thiourea, 0.002% 2-chloro-6-(trichloromethyl)pyridine, or 10^{-2} M potassium chlorate. Hydrazine inhibited the growth at a concentration of 10^{-3} M, but the inhibition was negligible at 10^{-4} M.

DISCUSSION

Nitrification by *Arthrobacter* sp. is striking in two notable ways: a number of novel nitrogenous compounds are formed, and these products are synthesized in relatively large amounts. Although the microbial formation of hydroxylamine by an oxidative pathway has been observed before (10, 12, 25), never were significant quantities released by actively replicating cells, and the identifications of the product were not rigorous. By contrast, the organism used in the present study excretes copious amounts of hydroxylamine, the identity of which was established unequivocally.

Many heterotrophic microorganisms have been reported to form nitrite from ammonium or amino groups, but the nitrite-nitrogen level is usually 0.5 to 2.0 μg/ml (11). *Arthrobacter* sp. is noteworthy because it forms far more of this anion. Certain fungi are known to synthesize nitrate from ammonium (8, 16), but the *Arthrobacter* strain investigated herein and the *A. globiformis* isolate studied by Gunner (10) are the only known bacteria generating nitrate from ammonium.

The results of various chemical tests, UV and infrared spectrometry, and mass spectrometry are consistent with the conclusion that the bound hydroxylamine compound is 1-nitrosoethanol. Nitroso compounds may exist as monomers or dimers in solution, and because the monomer-dimer equilibrium is readily reversible, it is usually difficult to tell whether an observed reaction should be attributed to a nitroso monomer or to its dimer (24). Therefore, although the compound is referred to herein as 1-nitrosoethanol, the biological product and the substance isolated may be either the monomer or dimer. To our knowledge, no aliphatic C-nitroso compound synthesized by a microorganism has been described. Because this compound is biologically novel, its chemical behavior has not been characterized, and a question remains as to whether the bacterium excretes the monomer or dimer, further chemical studies seem warranted.

A number of environmental factors markedly influence nitrification by *Arthrobacter* sp. Thus, the formation of hydroxylamine is relatively independent of pH in the neutral and alkaline range, whereas the formation of nitrite is favored by an alkaline reaction. The amount of ammonium relative to the quantity of the organic substrate, as well as the absolute quantity of the carbonaceous nutrient, contributes to the extent of nitrification. The iron concen-

tration in the medium also significantly affects the process. When no iron is supplied, nitrification is poor, and a hydroxamic acid is the major nitrification product. The inverse relationship between the amount of hydroxamic acid and the iron concentration of the medium resembles that reported for various bacteria and fungi (20). The inverse relation between the yields of hydroxamic acid and 1-nitrosoethanol might indicate that the nitrification process generates hydroxamic acid molecules mainly when an iron-chelating hydroxamate is needed to facilitate growth in iron-poor circumstances. When little of the iron-chelating agent is needed, nitrification is channeled to give rise to nitrite, 1-nitrosoethanol, or other oxidized nitrogenous products.

Among the chemicals known for their effective inhibition of autotrophic nitrification are 1-allyl-2-thiourea, hydrazine (6), 2-chloro-6-(trichloromethyl)pyridine (21), and chlorate (7). Of these, 1-allyl-2-thiourea, 2-chloro-6-(trichloromethyl)pyridine, and potassium chlorate, when used at concentrations which totally suppress autotrophic nitrification, had no effect on nitrification by *Arthrobacter* sp. The insensitivity of this bacterium to these three inhibitors further illustrates the physiological differences between heterotrophic and autotrophic nitrifiers.

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