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Heterotrophic Nitrification by *Arthrobacter sp.* (Strain 9006) as Influenced by Different Cultural Conditions, Growth State and Acetate Metabolism

Karl-Paul Witzel and Hans Jürgen Overbeck*

Max-Planck-Institut für Limnologie, Postfach 165, D-2320 Plön, Federal Republic of Germany

Abstract. An *Arthrobacter sp.* (strain 9006), isolated from lake water, accumulated nitrite up to about 15 mg N/l, but no nitrate. In a mineral medium supplemented with tryptone, yeast extract, acetate and ammonium, the cells released nitrite into the medium parallel to growth or when growth had virtually ceased. The nitrite formed was proportional to the initial acetate concentration, indicating an involvement of acetate metabolism with nitrification. The organism grew with a wide variety of organic carbon sources, but washed cells formed nitrite from ammonium only in the presence of citrate, malate, acetate or ethanol. Magnesium ions were required for nitrification of ammonium and could not be replaced by other divalent metal ions. Analysis of the glyoxylate cycle key enzymes in washed suspensions incubated in a minimal medium revealed that isocitrate lyase and malate synthase were most active during the nitrification phase. Nitrite accumulation but not growth was inhibited by glucose, tryptone and yeast extract. A possible explanation for the different nitrification patterns during growth is based on the regulatory properties of glyoxylate cycle enzymes.

Key words: *Arthrobacter sp.* – Heterotrophic nitrification – Ammonium – Nitrite – Acetate – Metal requirement – Isocitrate lyase – Malate synthase.

Besides the well known autotrophic nitrifiers belonging to the *Nitrosomonas* and *Nitrobacter* group, there are some heterotrophic microorganisms that are able to nitrify in the sense of a 'biological transformation of nitrogen in organic and inorganic compounds from a reduced to a more oxidized state' (Alexander et al., 1960). Among them are several fungi, especially *Aspergillus* and *Penicillium* species (Eylar and Schmidt, 1959; Doxtader and Alexander, 1966a) as well as actinomycetes (Hirsch et al., 1961; Doxtader and Alexander, 1966b) and bacteria (Fisher et al., 1956; Amarger and Alexander, 1968; Verstraete and Alexander, 1972a; Gode and Overbeck, 1972). Numerous organic and inorganic substances can be used as N-source for nitrification or are intermediates in an organic or inorganic pathway to the end products nitrite or nitrate (Focht and Verstraete, 1977). Most fungi excrete mainly nitrate (Eylar and Schmidt, 1959; Marshall and Alexander, 1962) in amounts of up to about 50 mg/l. However, the main nitrification product of bacteria and actinomycetes seems to be nitrite (Hirsch et al., 1961; Doxtader and Alexander, 1966b; Verstraete and Alexander, 1972a, b), although sometimes some nitrate formation was observed (Gunner, 1963; Verstraete and Alexander, 1972a).

In contrast to the autotrophic nitrifiers, most heterotrophs seem to excrete nitrite or nitrate only after the active phase of growth (Alexander et al., 1960; Doxtader and Alexander, 1966a; Obaton et al., 1968; Verstraete and Alexander, 1972a). This is the reason why several authors have suggested that heterotrophic nitrification might be associated with autolytic processes (Alexander et al., 1960; Marshall and Alexander, 1962) and does not contribute to the energy budget of the cells (Aleem, 1970).

Most bacterial isolates accumulated only low amounts of the nitrification products and often ceased to nitrify in the course of time. So, very little is known on the physiology of heterotrophic, nitrifying bacteria and the conditions leading to nitrite formation.

We have isolated an actively nitrifying *Arthrobacter sp.* (strain 9006) from lake water and tested the influence of some environmental factors and growth state on nitrite formation from ammonium.

* To whom offprint requests should be sent

Abbreviations Used. IL = Isocitrate lyase [*threo*-D_s-isocitrate glyoxylate-lase, E.C. 4.1.3.1]; MS = malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), E.C. 4.1.3.2.]

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Materials and Methods

Organisms and Culture Conditions. Arthrobacter sp. (strain 9006) was isolated from enrichments in F-medium (see below) which was inoculated with a water sample of the Kleiner Plöner See (Holstein, Germany) from 1 m depth on July 6th, 1970. It is available upon request and will be deposited at the Deutsche Sammlung von Mikroorganismen, Göttingen. Isolation procedure and description of the strain will be published in detail elsewhere.

Design of Experiments with Growing Cultures and Washed Cell Suspensions. Experiments with growing organisms were performed in shaken Erlenmeyer flasks (100 ml or 500 ml) containing 20 or 200 ml of F-medium at 200 rev/min and 27° C. The F-medium contained (g/l distilled water): 5.0 sodium acetate · 3 H_2O; 0.2 tryptone; 0.2 yeast extract; 0.3 NH_4Cl; 0.1 $NaHCO_3$; 0.6 $MgSO_4$ · 7 H_2O; 0.01 $CaCl_2$ and 1 ml of Hoagland's trace elements solution. It was autoclaved for 20 min at 121° C, and separately autoclaved phosphate buffer pH 7.4 was added to a final concentration of 50 mmol/l.

For experiments with washed cell suspensions, the cells were grown in shaken cultures with 200 ml F-medium, harvested under sterile conditions in the second half of the growth phase by centrifugation (20 min at 5000 × *g*, 4° C), washed twice with 0.05 molar TRIS/HCl pH 7.4, and resuspended in the same buffer so that the cell concentration was about 20 times that in the growing culture. Efficiency of washing was checked by nitrite determination in the final cell suspension. To start the experiments, 1 ml of this suspension was added to 19 ml of autoclaved minimal medium which consisted of (g/l distilled water): 5.0 sodium acetate · 3 H_2O; 0.3 NH_4Cl; 0.6 $MgSO_4$ · 7 H_2O; and 50 mmol phosphate buffer pH 7.4. Deviations from this composition are indicated in the text. Thus, initial cell density was about the same as in growing culture. The cell suspension was incubated in 100 ml Erlenmeyer flasks using three replicates and appropriate controls on a rotary shaker at 27° C, mostly for 20 – 40 h as indicated. After this time, 1 ml of the suspension was withdrawn for determination of total protein content. The rest was centrifuged at 5000 × *g* for 40 min, and the supernatant used for the determination of dissolved protein, ammonium, nitrite, nitrate, and acetate. Cell protein content was calculated as the difference between total and dissolved protein.

Analytical Procedures. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (Cohn Fr. V) as a standard. For dry weight determinations, 10 ml of a culture were centrifuged in glass tubes at 5000 × *g* for 40 min, the pellet washed once with distilled water and dried to constant weight at 85° C. Optical density of the suspensions was determined using an Eppendorf photometer at 623 nm in cuvettes with 1 cm light path. Ammonium analysis was performed by the indophenol method. A given volume of the culture supernatant was mixed with 5 ml of a solution containing 85 g sodium salicylate, 200 g sodium hydroxide, 0.6 g sodium nitroprusside per liter. After 5 min in the dark, 5 ml of a 0.2% (v/v) sodium dichlorcyanurate solution were added and the sample made up to 100 ml. After 90 min in the dark, extinction was measured in a Zeiss Elko II photometer with filter I 61.9 and light path of 1 or 0.5 cm, using a blank with distilled water treated as above. To determine nitrite concentrations, 5 ml of the culture supernatant or an appropriate dilution of it in distilled water were mixed with 0.5 ml of a freshly prepared reagent containing 100 ml *ortho*-phosphoric acid (min. 85%), 40 g sulfanilamide, 2 g N-1-naphthylethylenediamine dihydrochloride in 1 l of distilled water. Extinction was measured at 530 nm after 10 min against a blank with distilled water treated as above. Nitrate was determined as nitrite after reduction with hydrazine sulfate and Cu^{2+} in a Technicon autoanalyzer, or according to the method of Czensny (1961) with extinction measurements in a Zeiss Elko II photometer with filter S 42. Acetate was determined enzymatically by the method of Holz and Bergmeyer (1970).

Preparation of Cell-Free Extracts and Enzyme Assays. For enzyme assays, the cells from batch cultures were harvested by centrifugation (8000 × *g*, 15 min, 1° C), washed twice with TRIS/HCl buffer (0.05 mol/l, pH 7.4) and resuspended in 5 ml of the same buffer. The cells were disrupted by sonication in a MSE Ultrasonic Power Unit 60 W model 3000 for 20 min at maximum capacity with cooling in ice. Temperature of the extract did not exceed 5° C. Cell debris were removed by centrifugation at 35000 × *g* and 1° C for 45 min. Isocitrate lyase was determined by the method of Dixon and Kornberg (1959), malate synthase by the method of Flavell and Fincham (1968).

All chemicals used were of highest purity available, enzymes and enzyme substrates were purchased from Boehringer, Mannheim.

Results

Nitrification and Growth

Arthrobacter sp. (strain 9006) growing in F-medium, accumulated nitrite but no nitrate. Growth in a mineral salts solution with a defined organic carbon source was not observed unless several amino acids were added as growth factors. In a complex medium containing peptone and meat extract without added ammonium and an extra carbon source, the organism grew, but did not accumulate nitrite nor nitrate. Nitrite accumulation in F-medium was suppressed by increasing tryptone and yeast extract concentrations. No nitrite was released in F-medium when acetate was omitted or when its concentration was below a certain threshold.

Growth, nitrite production and ammonium uptake were, therefore, followed in F-medium with two different concentrations of acetate: one favoring active nitrification (880 mg C/l; Fig. 1), the other just at the threshold (88 mg C/l; Fig. 2). All other constituents of F-medium remained unchanged. Both the media were inoculated with 1 ml of a culture in nutrient broth per 200 ml F-medium. The organisms grew under both conditions nearly equally well; the yield of cell protein was not markedly altered by acetate concentration. Parallel to growth, extracellular protein decreased by about the same amount as cellular protein increased. Under similar conditions, acetate concentration did not decrease significantly until all extracellular protein had been used up (results not shown). During the utilization of tryptone and yeast extract, ammonium accumulated in the medium up to about double the initial value. With high initial acetate concentration, nitrite accumulation was paralled by ammonium utilization, the release of an extracellular protein, and increasing total protein content, whereas cell protein and optical density remained constant. The ammonium nitrogen taken up and the nitrite nitrogen excreted were not stoichiometric, resulting in a comparatively low efficiency of nitrification, although the freshly synthesized extracellular protein might account for some of the lack. In contrast, when acetate was provided in a limiting

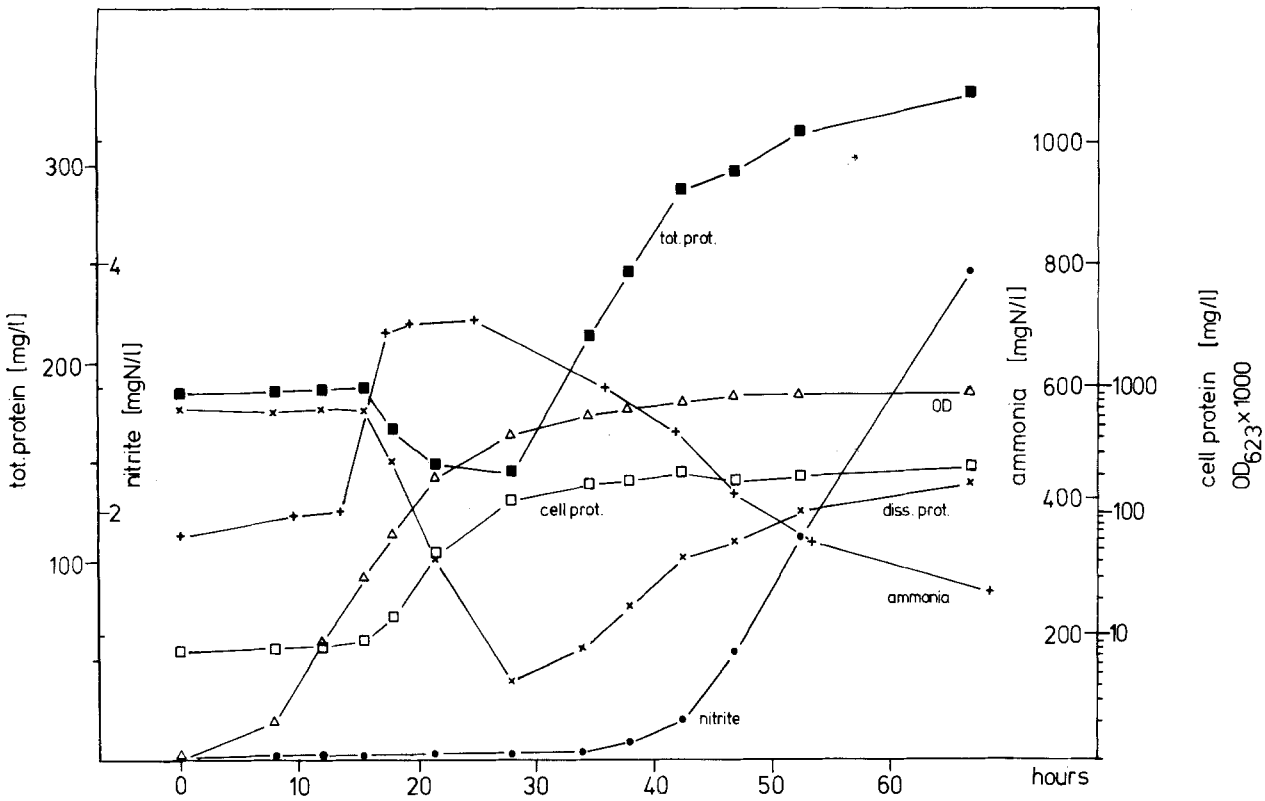

Fig. 1. Growth and nitrite accumulation by *Arthrobacter sp.* in F-medium with the normal acetate concentration (880 mg C/l). 200 ml of the medium were inoculated with 1 ml of an overnight culture in nutrient broth. Growth parameters, nitrite and ammonium were analysed as described in "*Materials and Methods*". □ cell protein; × extracellular protein; ■ total protein; + ammonium; △ optical density

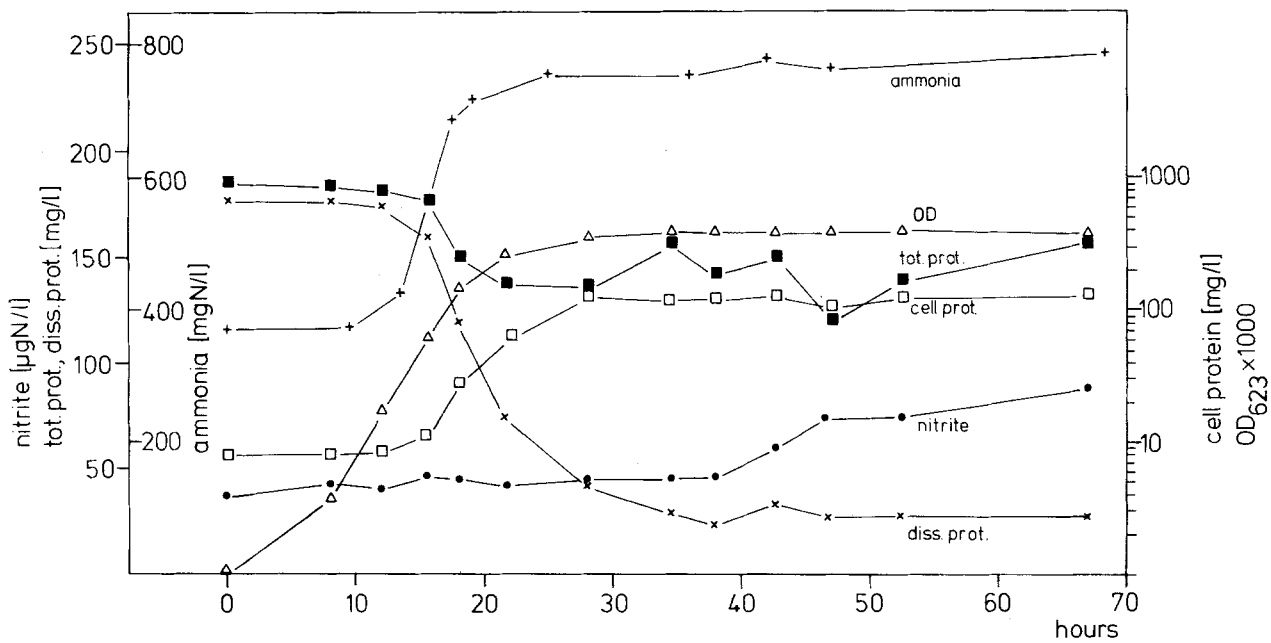

Fig. 2. Growth and nitrite accumulation by *Arthrobacter sp.* in F-medium with 1/10 of the normal acetate concentration (88 mg C/l). Per formance of the experiment and symbols used as in Fig. 1

concentration, neither nitrite nor extracellular protein was released, and ammonium was not taken up.

When F-medium was inoculated with a culture in the same medium in which the cells had been induced to acetate metabolism (Fig. 3), nitrite accumulated already during active growth; dissolved and total protein concentrations increased parallel to growth and were obviously linked to the beginning of nitrite accumulation.

Effect of Different Carbon Sources

Nitrite formation from ammonium was strongly influenced by acetate concentration, showing an optimum at about 900 mg C/l in growing cultures and about 500 mg C/l in washed cell suspensions.

Without an organic carbon source, nitrite did not accumulate to a level higher than about 20 µg N/l when ammonium was the nitrogen source. Citrate, malate and ethanol could substitute for acetate, but glucose, fructose, lactose, pyruvate, lactate, 2-oxoglutarate, succinate, glyoxylate, formate, palmitate, stearate, glycerol or methanol at concentrations of 880 mg C/l could not. The highest amounts of nitrite were found with citrate, and malate also gave higher nitrite yield than did acetate.

Glucose, when added to washed cell suspensions in minimal medium with acetate even had an inhibitory effect on nitrification. While cell yield (as dry weight) was stimulated by higher glucose concentrations, nitrite formation was depressed already by about 80% at 1 mmol glucose per liter. A similar inhibition was caused by tryptone and yeast extract provided to washed cell suspensions. Nitrification was inhibited to about 50% by 200 mg/l each of tryptone and yeast extract, whereas the formation of cell protein was stimulated and extracellular protein concentration remained unaffected.

Effect of Divalent Metal Ions

In growing cultures and washed cell suspensions, nitrification of ammonium or amino acids was observed only in the presence of Mg^{2+}, no matter what the associated cations had been, and which of the suitable carbon sources had been provided. In washed cell suspensions, nitrification activity was only slightly affected by varying the magnesium concentration between 10 µmol/l and 2.4 mmol/l. But below, it sharply decreased with decreasing magnesium concentrations. Magnesium ions could not be replaced by Mn^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+} or Cu^{2+} (as sulfates at 2.5 mmol/l). With $CoSO_4$, a nitrite production four times higher than the control, but only 1/15 of that with magnesium was observed.

Nitrification and Acetate Metabolism

Further evidence for relationships between acetate metabolism and nitrification was obtained by enzy-

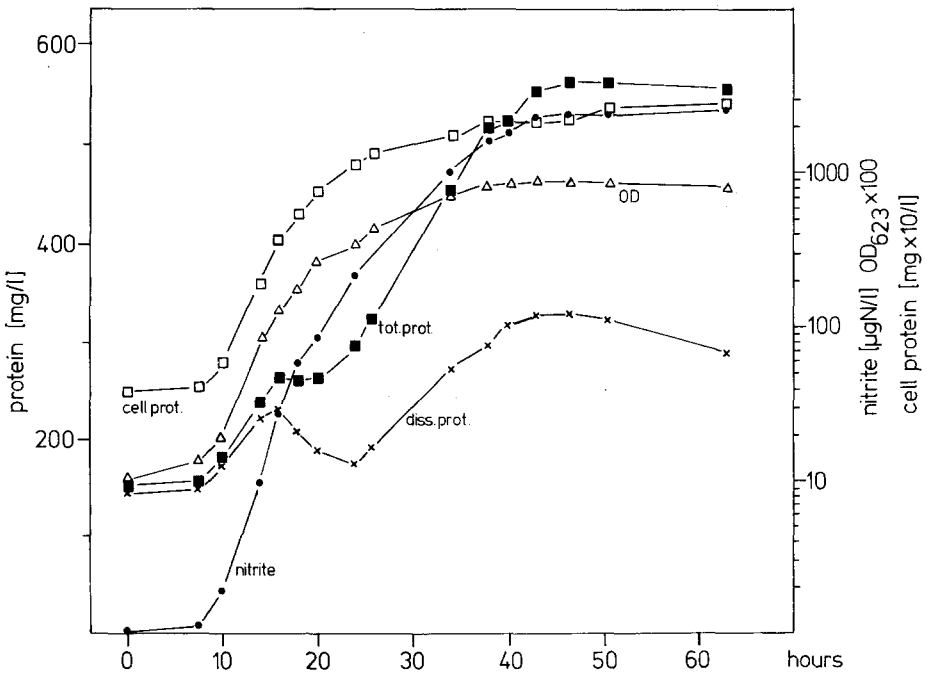

Fig. 3
Growth and nitrite accumulation by *Arthrobacter sp.* in F-medium inoculated with 1 ml of a 24 h culture in F-medium per 200 ml. Performance of the experiment and symbols used as in Fig. 1

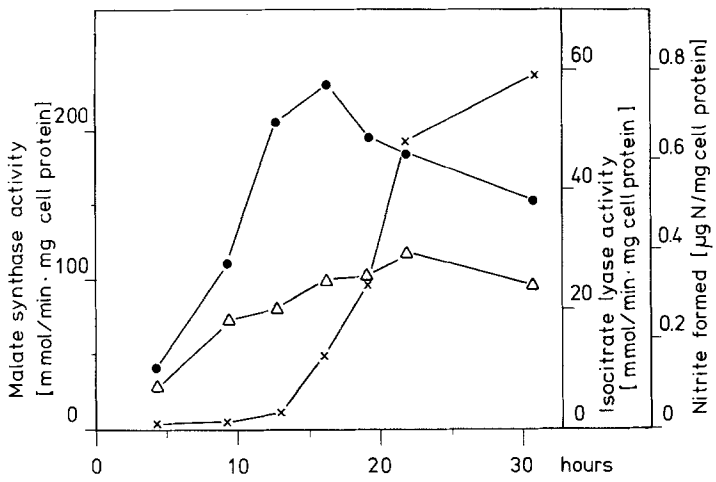

Fig. 4
Activities of the glyoxylate cycle key enzymes [isocitrate lyase (*IL*), malate synthase (*MS*)] and nitrite accumulation in a washed cell suspension of *Arthrobacter sp.* in minimal medium. Enzyme activities and nitrification are related to cell protein of the sample. × nitrification; ● isocitrate lyase; △ malate synthase

matic analysis of the relevant metabolic pathway. Activities of the glyoxylate cycle key enzymes isocitrate lyase [*threo*-D_s-isocitrate glyoxylate-lyase, E.C. 4.1.3.1], and malate synthase [L-malate glyoxylate lyase (CoA-acetylating), E.C. 4.1.3.2] in relation to the nitrification activity of washed cells are shown in Fig. 4. After resuspending the washed cells in minimal medium (containing acetate, ammonium, magnesium and phosphate buffer), the nitrification activity (measured by the amount of nitrite released between two samplings and corrected for length of incubation and cell protein) had a lag phase of about 10 h. During this period, the activities of the two key enzymes increased, both having a maximum during nitrite excretion. The maximum of isocitrate lyase activity was followed by that for malate synthase with a delay of about 7 h.

Discussion

Nitrification by heterotrophic microorganisms seems to differ in four main points from autotrophic nitrification: (1) a broad spectrum of organisms, N-sources and products are involved in heterotrophic nitrification (Hirsch et al., 1961; Focht and Verstraete, 1977); (2) an organic carbon source is necessary not only for growth of the organisms but in addition for nitrification of at least inorganic N-sources (Hylin and Matsumoto, 1960; Verstraete and Alexander, 1972b); (3) heterotrophic nitrifiers are much less active and effective in their nitrification ability than autotrophic ones (Gode and Overbeck, 1972; Focht and Verstraete, 1977); (4) the heterotrophs mostly accumulate nitrite or nitrate when active growth of the cells has ceased (Alexander et al., 1960; Doxtader and Alexander, 1966c; Obaton et al., 1968; Verstraete and Alexander, 1972a). These latter two points have led to the assumption that nitrite production by heterotrophs is a passive process linked to lysis of the cells (Alexander et al., 1960; Marshall and Alexander, 1962).

The results presented here, however, show that nitrite excretion is a distinct metabolic feature of *Arthrobacter sp.* (strain 9006). The clear dependency of nitrite accumulation on certain carbon sources and specific divalent metal ions is strong evidence that metabolically active cells are required for nitrification. So, it can be readily assumed that nitrite is formed from ammonium by an oxidative sequence involved in some way with the metabolism of a special carbon source, and that at least one step is Mg^{2+}-dependent.

Metal requirements for nitrification are known in the case of *Nitrosomonas* (Loveless and Painter, 1968) which requires Mg^{2+}, whereas some heterotrophic nitrifiers are stimulated by Fe^{2+} and Fe^{3+} (Aleem et al., 1964; Verstraete and Alexander, 1972a) which may be replaced by several other metal ions. It seems that strain 9006 is the only heterotrophic nitrifier with a specific need for Mg^{2+}. At the moment, an explanation for this marked requirement is available only on the basis of the known Mg^{2+}-dependency of both the glyoxylate cycle key enzymes (see below).

The function of the carbon source is up to now not quite clear. But, assuming an organic pathway of nitrogen oxidation (Alexander et al., 1960; Verstraete and Alexander, 1972b), a specific carbon source could provide the acceptor molecules for the synthesis of intermediate organic N-compounds. If the carbon skeleton is not recycled, there should be a relationship between the amount of carbon utilized and the nitrite released; and there should be any organic by-product formed from the carbon source during nitrification. In fact, the results indicate that acetate was metabolized via the glyoxylate cycle during nitrite excretion (Fig. 4), the amount of nitrite accumulated was proportional to

the initial acetate concentration (Figs. 1, 2), and an organic substance reacting with the Folin-reagent was released parallel to nitrification (Fig. 1). However, there is up to now no convincing explanation why nitrite is produced only with acetate, citrate, malate, and ethanol while several other C-sources supported growth but not nitrification. Similar carbon dependencies of heterotrophic nitrification have been reported (Hylin and Matsumoto, 1960; Verstraete and Alexander, 1972 b; O'Neill and Wilkinson, 1977), but the carbon sources favoring nitrification are not unique among different organisms. However, our results are roughly in accordance with that of Verstraete and Alexander (1972 b) working with the same genus.

Nitrification by *Arthrobacter sp.* (strain 9006) growing in F-medium can proceed equally well during or after growth, depending on the cultural conditions for the inocula. This may be explained by the regulative properties of the glyoxylate cycle key enzymes (Kornberg, 1966) which are induced by acetate and may be repressed by glucose, PEP, or pyruvate (Donawa and Inniss, 1970; Kleber and Müller, 1970; Hanozet and Guerritore, 1972; Wolfson and Grulwich, 1972). Extracts of *Arthrobacter sp.* (strain 9006) contained malate synthase (MS) and isocitrate lyase (IL), the activity of which was induced by incubation in a medium containing acetate. Moreover, in the washed cell suspensions isocitrate lyase had its maximum activity at the beginning of, malate synthase during the nitrification phase, indicating a metabolization of acetate parallel to nitrite formation. So, it seems likely that cultures growing in F-medium inoculated with uninduced cells from nutrient broth, accumulate nitrite only when tryptone and yeast extract are used up (as shown in Fig. 1), because acetate metabolism is repressed during growth. By contrast, cultures inoculated with induced cells are adapted to metabolize acetate parallel to growth with tryptone and yeast extract, and so can accumulate nitrite parallel to growth, but to a lesser extend (Fig. 3). The inhibition of nitrite accumulation by glucose and tryptone/yeast extract in washed cell suspensions does support this explanation. The fact that nitrification can proceed parallel to growth does not necessarily indicate any causality between growth and nitrification, because in F-medium acetate does not mainly contribute to the formation of cell mass.

It remains to be tested whether this type of nitrification pattern observed with *Arthrobacter sp.* (strain 9006) growing in F-medium may be specially valid only for this organism and in the medium used. But the present study opens some insight into the role of the carbon in heterotrophic nitrification by *Arthrobacter sp.*, and demonstrates clearly that nitrification is integrated into the cell metabolism, especially the transformation of special carbon sources.

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