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Growth of *Nitrobacter* in the Presence of Organic Matter

II. Chemoorganotrophic Growth of *Nitrobacter agilis*

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Abstract. 1. After a resting period of up to 6 months cells of *Nitrobacter agilis* grow with acetate, formate, and pyruvate as carbon and energy source. Yeast extract and peptone were added to supply the organism with nitrogen and to meet possible vitamin requirements.

2. The length of the growth period depends on the substrate; it increases according to the following sequence: pyruvate, formate, acetate. The highest growth yield is observed with pyruvate, the lowest with formate.

3. O_2 consumption is increased in the presence of substrates as compared to endogenous respiration. With pyruvate and acetate twice as much O_2 is consumed, with formate 7 times, with yeast extract-peptone 10 times as much.

4. The ability of nitrite oxidation is largely preserved, except in cells grown with acetate or pyruvate in the presence of 0.015% yeast extract and peptone. Such cells have nearly no cytochrome a_1. Accordingly, the cytochrome spectra of nitrite oxidizers grown under chemoorganotrophic and lithoautotrophic conditions coincide qualitatively.

5. The nitrite oxidizing system is inducible. It is induced by nitrite but also by substances present in yeast extract and peptone. Cells grown on acetate and yeast extract and peptone (0.015%) require 3–4 weeks before they regain the ability to grow with nitrite. Cells grown chemoorganotrophically with the same substrates and yeast extract and peptone (0.15%) start growing with nitrite as energy source without a lag.

6. Cell size and form, distribution of storage materials, order and fine structure of double membranes are correlated with growth conditions.

Key words: *Nitrobacter agilis* – Chemoorganotrophic growth – Acetate – Formate – Pyruvate – Yeast extract-peptone – Ultrastructure.

There have been many attempts to grow nitrifying bacteria which according to Winogradsky (1888) have to be regarded as obligately lithoautotrophic organisms in the presence of organic substances. Smith and Hoare (1968) proved that *Nitrobacter agilis* can grow with acetate and hydrolyzed casein. Under such conditions fine structure and metabolism are almost identical in cells grown autotrophically or heterotrophically (Pope et al., 1969). Possibly acetate can be replaced by formate (van Gool and Laudelout, 1966; O'Kelley and Nason, 1970).

This study describes a strain of *N. agilis* capable of growing heterotrophically with acetate, pyruvate, formate, and yeast extract-peptone. As revealed by morphological investigations some fine structures change with the substrate while others remain unchanged.

MATERIALS AND METHODS

Organism. For chemoorganotrophic growth experiments the nitrite oxidizing bacterium *Nitrobacter agilis* (Westphal and Bock, 1974) previously described as *Nitrobacter* K_1 (Bock et al., 1974) was used.

Culture Conditions. The organism was grown in liquid media containing in 1000 ml of distilled water the following minerals: NaCl 0.5 g, $MgSO_4 \cdot 7\,H_2O$ 0.05 g, KH_2PO_4 0.15 g, $CaCO_3$ 0.07 g, $(NH_4)_6MO_7O_{24} \cdot 4\,H_2O$ 50 µg, $FeSO_4$ 150 µg. Acetate, formate, pyruvate or α-ketoglutaric acid, respectively, were added, each 1.0 g, as source of carbon and energy. Yeast extract (Difco) and peptone (Difco), 0.15 g each together with acetate, pyruvate or α-ketoglutarate; 1.5 g each with formate or pyruvate, was supplied as source of nitrogen. Cresol-red was used as pH indicator. After

Abbreviations. CM = cytoplasmatic membrane; YP = yeast extract-peptone

Table 1. Adaptation and growth of *Nitrobacter agilis* in the presence of organic substrates. The concentrations of yeast extract-peptone (YP) are given as g/l

	YP (0.15, 1,5, 3.0)	Pyruvate + YP (1.5)	Formate + YP (1.5)	Acetate + YP (0.15)	α-Ketoglutarate + YP (0.15)
Adaptation period (weeks)	8	8	8	24	30
Growth period (weeks)	5	3	7	8	20
	(0.15) (1.5) (3.0)				
Cell yield (μg N/l)	< 200 370 1100	10000	1100	2200	< 200

sterilization (121° C, 30 min) the pH value was 7.4–7.5. If by consumption of the substrate the pH value increased beyond 7.8 it was adjusted to 7.4 by addition of 0.6 N H_2SO_4.

Cultures were inoculated by addition of 2.5–5% (v/v) of a suspension which had been grown mixotrophically with nitrite and 0.15% yeast extract-peptone (Steinmüller and Bock, 1976). For the purpose of adaption the cultures (100 or 600 ml) were held in the incubator at 28° C. Growing cultures were stirred at 28° C by means of a magnetic stirrer (cultures volume 2500 ml).

Every culture was checked for contamination by inoculation of 4 different complex agar media and of 1 complex liquid medium. The agar media contained in % (w/v): (I) yeast extract, 0.5; peptone, 0.5; KH_2PO_4, 0.015; $MgSO_4 \cdot 7\,H_2O$, 0.005; NaCl, 0.05; (II) yeast extract, 0.15; peptone, 0.15; pyruvate, 0.1; KH_2PO_4, 0.015; $MgSO_4 \cdot 7\,H_2O$, 0.005; NaCl, 0.015; (III) yeast extract, 0.15; peptone, 0.15; acetate, 0.1; KH_2PO_4, 0.015; $MgSO_4 \cdot 7\,H_2O$, 0.005; NaCl, 0.05; (IV) yeast extract, 0.15; peptone, 0.15; formate, 0.1; KH_2PO_4, 0.015; $MgSO_4 \cdot 7\,H_2O$, 0.005; NaCl, 0.05; Bacto agar (Difco) 1.5 each. The liquid medium at portions of 5 ml per flask contained in % (w/v): nutrient broth (Difco), 0.3; yeast extract, 0.15; KH_2PO_4, 0.015; $MgSO_4 \cdot 7\,H_2O$, 0.005; NaCl, 0.05. The plates and flasks were incubated for 2 weeks at 30° C and examined for colonies respectively turbidity. In liquid medium no growth could be observed. The agar plates did not give rise to colonies even if they were examined by means of a stereomicroscop. After 3 weeks only on pyruvate plates tiny colonies (< 0.05 mm) could be observed. Such colonies consisted of some 100 cells which were identical in morphology to those grown on pyruvate medium. Transferred to mineral nitrite medium every colony gave rise to nitrification. In addition pyruvate culture suspensions of about 5×10^8 cells (determined by direct counting) were diluted and inoculated of nitrite medium. They also gave rise to nitrification up to a dilution state of $1 : 10^8$.

Chemoorganotrophic and mixotrophic cells were examined with the light microscope. Generally more than one culture grown with following substrates was embedded in Epon: Pyruvate (3×), acetate (4×), formate (2×), nitrite + yeast extract-peptone = mixotrophic cells (2×), yeast extract-peptone (1×) and α-ketoglutarate (1×). Ultrathin sections were checked for morphological characteristic by use of an electron microscope (Siemens, Elmiscope I).

Electron Microscopy and Analytical Techniques. The electron microscopical methods were those described by Bock and Heinrich (1969). Photographs of living cells were taken by use of the photomicroscope II (Zeiss, phase contrast, Neofluar, 100/1.30). Measurements of O_2 consumption were done by the conventional Warburg technique. Difference absorption spectra were determined in 1 cm quartz cuvettes using the Shimadzu UV 200 double beam spectrophotometer with integrating sphere.

RESULTS

1. Adaptation, Growth, and Growth Yield

In order to allow adaptation to organic energy substrates cells from lithoautotrophic or mixotrophic cultures were inoculated into solutions containing different organic substances. Yeast extract-peptone was supplied as source of nitrogen and vitamins. It was added in low concentrations of 0.15% or 0.015% (Table 1).

Cell yield and respiratory activity were determined in cultures growing in the third passage on organic media. As shown in Table 1, the period of adaptation and growth depends on the nature of the substrate. The bacterial yield, determined as cell-N, after 3 weeks of growth on pyruvate, was 10 mg per liter. This yield was 4.5 times higher than that obtained after 8 weeks' growth with acetate, and 9 times higher than that obtained after 7 weeks on formate or 5 weeks on yeast extract-peptone (3 g/l). In cultures with α-ketoglutarate there was but a minimal cell multiplication although microscopic examination revealed about 10^6 bacteria/ml after the third passage. As shown by electron microscopic studies these cells possessed the characteristic features of *Nitrobacter* (Fig. 5). After inoculation into a mineral salt solution containing nitrite all the cells grown chemoorganotrophically were capable of oxidizing nitrite.

2. Endogenous Respiration and Substrate Respiration

Immediately after use-up of nitrite the resting cells of *Nitrobacter* showed a characteristic decrease of their endogenous respiration. The drop in activity started already 1 h after exhaustion of nitrite and arrives at 1/10 of the original activity after 6 days (Fig. 1). The cells were not yet dead; the decrease in viability started not earlier than 3 months (Schrader, personal communication). Cells grown with nitrite plus acetate which had deposited much PHB exhibited a constant

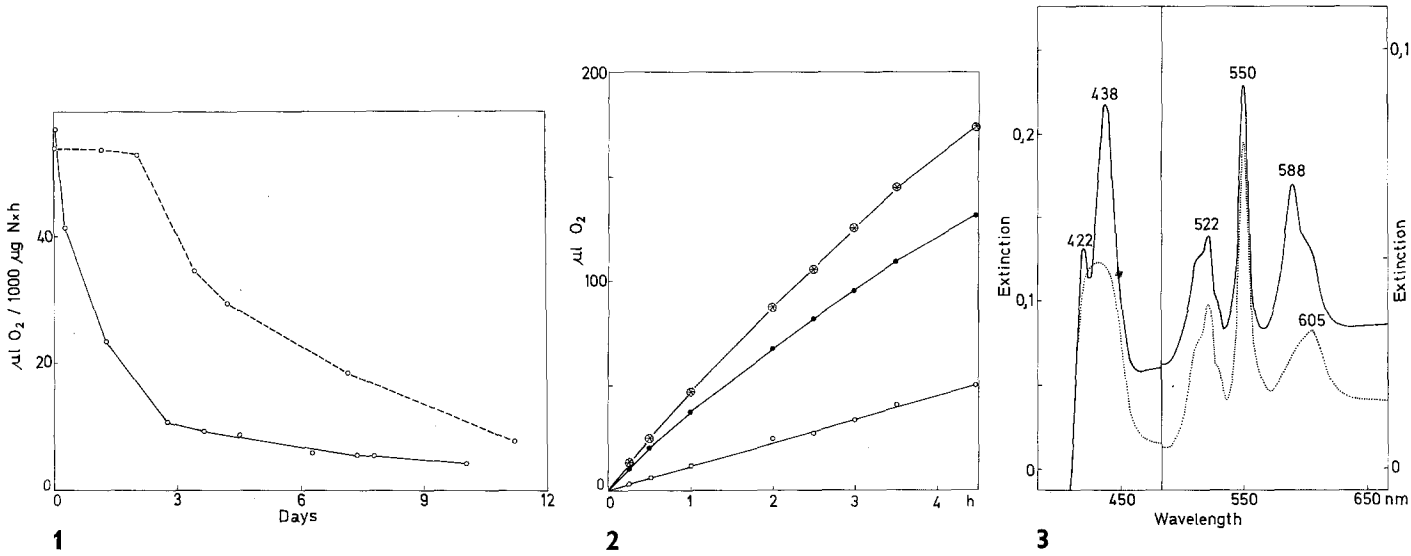


Fig. 1. Decrease of endogenous respiratory activity following nitrite oxidation by *Nitrobacter agilis*. In the Warburg vessel: 2.0 ml of bacterial suspension. In the center well: 0.1 ml of KOH (10%). O——O cells grown with nitrite; O------O cells grown with nitrite + acetate

Fig. 2. Endogenous and substrate respiration of *Nitrobacter agilis*. In the Warburg vessel: 2.0 ml of bacterial suspension, equivalent to 568 µg N. Substrate: 0.1 ml YP. Center well: 0.1 ml of KHO (10%). O——O endogenous respiration; ●——● respiration in the presence of 6 mg yeast extract-peptone; ⊛——⊛ respiration in the presence of 12 mg yeast extract-peptone

Fig. 3. Difference absorption spectra of heterotrophically grown cells of *Nitrobacter agilis*. The figures describe the position of the peaks. —— cells growth with pyruvate and 0.15% YP (1159 µg N/ml); ------- cells grown with acetate and 0.015% YP (710 µg N/ml). Reduction with Na-dithionite

Table 2. Endogenous and substrate respiration of *Nitrobacter agilis* and *Escherichia coli*. Concentration of the nitrogen source is given in brackets (as g/l). Figures for respiratory activity are mean values (µl O_2/mg N × h)

	Nitrobacter agilis							*Escherichia coli*
Substrate	Nitrite	Nitrite + YP (1.5)	YP (3.0)	Pyruvate + YP (0.15)	Pyruvate + YP (1.5)	Formate + YP (1.5)	Acetate + YP (0.15)	Tryptone
Endogenous respiration	54	18	19	30	28	16	35	30[a]
Substrate respiration	–	–	60	95	70	109	71	370[b]
Nitrite oxidation	4000	19000	730	150	1400	800	61	–

[a] Rittenberg and Shilo (1970)
[b] Gadkari (personal communication)

respiratory activity for approx. 2 days. Then, the decrease was slower as compared to cells grown with nitrite only (Fig. 1).

The course of endogenous respiratory activity of nitrite oxidizers grown chemoorganotrophically corresponded to the latter type described. Cells grown with yeast extract-peptone, pyruvate, formate, or acetate had a constant O_2 consumption during a period of $4^1/_2$ h. The activity per cell equaled that of a heterotrophic bacterium such as *Escherichia coli* (Table 2).

In the presence of yeast extract-peptone the O_2 consumption increased up to 8–10 times depending on the concentration (up to 20 g/l). As shown in Figure 2 the O_2 consumption in the presence of 3.0 g or 6.0 g yeast extract-peptone per 1 is 2 times or 3 times, respectively, that of endogenous respiration. However, it did not stay constant, but decreased for 1/3 of the initial value after $4^1/_2$ h. Supply of pyruvate or acetate was followed by an increase in O_2 consumption to 2–3 times the amount of endogenous respiration; with formate, the O_2 consumption was 7 times that of endogenous respiration (Table 2).

Nitrite oxidizers grown heterotrophically were different with respect to the activities of nitrite oxidation (Table 2). Compared to the activity of litho-

autotrophic cells the bacteria grown on yeast extract-peptone (YP), or formate + 0.15% YP, or pyruvate + 0.15% YP retained but 20–30% of activity. The activity of cells grown with acetate + 0.015% YP or pyruvate + 0.015% YP was much less (1–3%). Cells grown mixotrophically with yeast extract-peptone and nitrite exhibited 5 times the activity of autotrophic bacteria.

3. *Cytochrome Spectra*

The cytochrome spectra of *Nitrobacter* have characteristic absorption bands for cyt. *c* and a_1. Since cyt. a_1 probably is an essential component of nitrite oxidation (Aleem, 1968) chemoorganotrophically grown cells were examined particularly with respect to this respiratory enzyme system. The difference spectra of cells grown with pyruvate + 0.15% YP revealed (Fig. 3) that cyt. *c* and cyt. a_1 with the α-bands at 550 nm and 590 nm, the β-bands at 522 mm, and the γ-bands at 420 nm and 438 nm were present in relatively high concentrations. This spectrum was very similar to that of lithoautotrophic cells. Only the ratio of cyt. a_1 to cyt. *c* is somewaht less in comparison to data given by Bock and Heinrich (1969) for lithoautotrophic nitrite oxidizers. The difference spectra of cells grown with yeast extract-peptone and formate were very similar. The difference spectra of acetate cells grown in the presence of 0.015% yeast extract-peptone lacked the bands of cyt. a_1 at 590 nm and 438 nm (Fig. 3). They had instead a peak at 605 nm (α-band of cyt. a_3) and a broad band between 428 nm and 440 nm. According to the spectrophotometric studies it was likely that acetate-grown cells had nearly no cyt. a_1.

4. *Ultrastructure*

Cell size and morphology of *Nitrobacter* changed with growth conditions. In general the size of cells grown with formate and α-ketoglutarate varied from 0.5–0.8 × 0.6–1.0 µm; cells from yeast extract-peptone measured 0.4–0.8 × 0.5–1.6 µm, and cells from acetate measured 0.6–0.9 × 0.8–1.7 µm. They were short rods. Cells grown with pyruvate were short rods of 0.5–0.9 × 0.7–1.5 µm, or long rods measuring 0.8 × 1.5–3.4 µm. Alterations in morphology caused by budding had been observed in cultures from acetate and pyruvate. In cultures supplied with pyruvate some bacteria were motile.

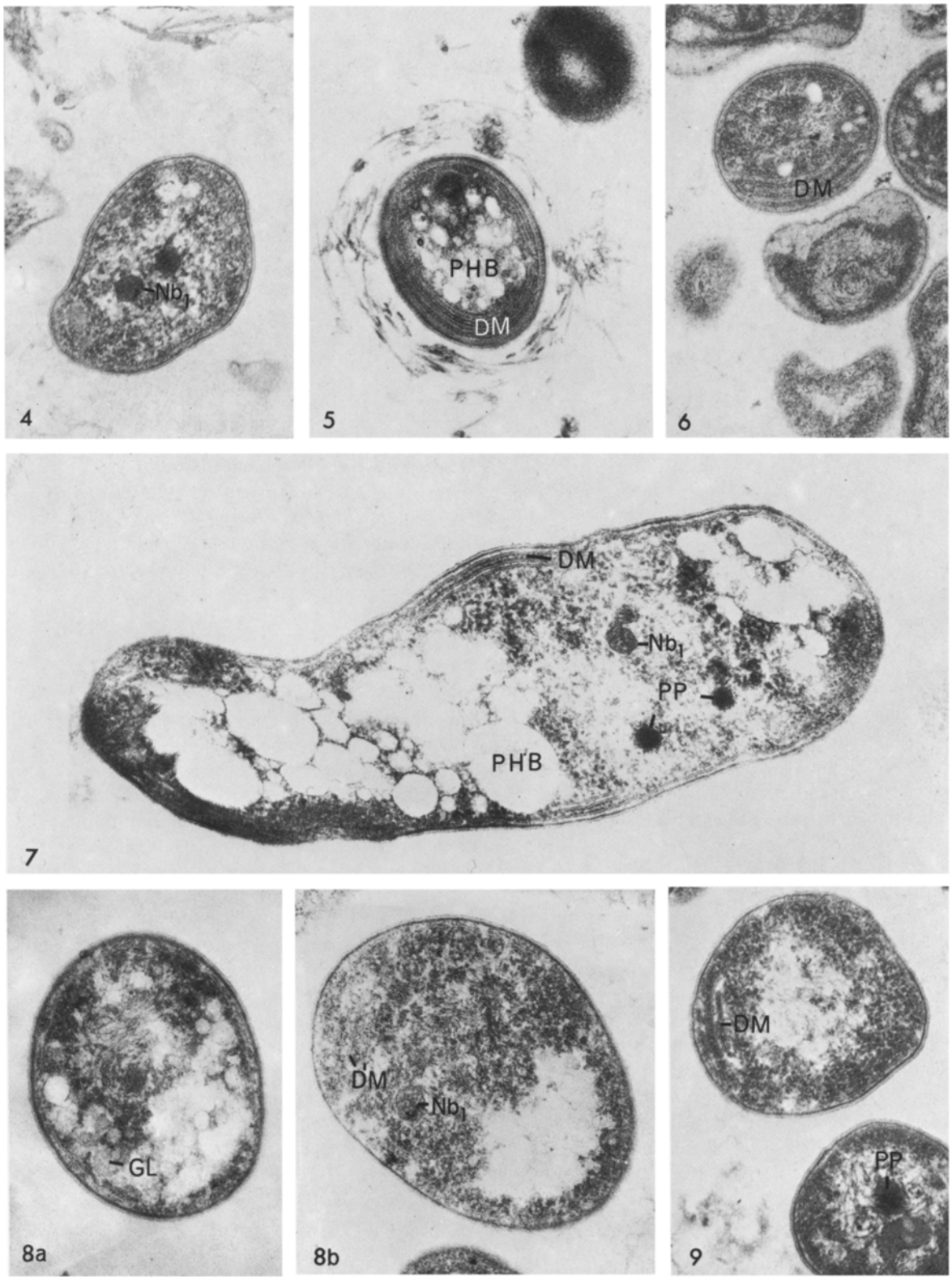
Ultrathin sections revealed (Figs. 4–9) that the structure of the cell wall of chemoorganotrophically grown bacteria was identical. The wall has 3 layers, and the zone adjacent to the interior of the cell strongly absorbed electrons. The structure was also identical with that of mixotrophic nitrite cells (Fig. 12a and b).

Cells grown with yeast extract-peptone, α-ketoglutarate, formate, and pyruvate (Figs. 5–7) are characterized by intracytoplasmic double membranes which may be located at one polar end (Fig. 6). Between two single membranes there was always an electron dense layer (Figs. 5–7). Cells grown with yeast extract-peptone have stored large quantities of PHB, polyphosphates, and possibly glycogen-like materials. In cells from α-ketoglutaric acid, formate, and pyruvate PHB granules prevailed (Figs. 5–7). Some cells from α-ketoglutaric acid in addition possed a thick slime layer (Fig. 5). In cells grown with acetate the CM and the double membranes were difficult to detect (Fig. 8a and b). There was no electron dense layer at the interior of the CM and between the double membranes. Although less well arranged, there were polarly located membranes in some cells (Fig. 8b). Storage granules prevailed which probably represented glycogen-like inclusions (Fig. 8a). In less than 5% of all ultrathin sections of heterotrophic *Nitrobacter* cells polyhedral particles had been found (Figs. 4, 7, and 8b) which had been characterized by Bock et al. (1974) as bacteriophage-like particles Nb_1.

5. *Induction of Nitrite Oxidase*

Since both aged (Eigener and Bock, 1972) and heterotrophically grown cells of *Nitrobacter* (Smith and Hoare, 1968) retained a relatively high activity of nitrite oxidation nitrite oxidase was supposed to be a constitutive enzyme. This view was in agreement with the results obtained with cells grown with yeast extract-peptone alone or pyruvate and formate with 0.15% yeast extract-peptone. After transfer into mineral solution the cells immediately reactivated their nitrite oxidizing system. After 14 days the added nitrite had been consumed.

The acetate-cells grown in the presence of 0.015% yeast extract-peptone did not agree with this conception as they practically were free of nitrite oxidase. After transfer of such cells into a mineral medium containing nitrite, 4 weeks passed before nitrification started. Cultures in acetate plus nitrite started one week earlier. Sections of such cells show structures (Fig. 9) which were intermediate between acetate cells (Fig. 8a and b) and nitrite cells (Fig. 12a and b). It can be assumed that cells having an electron dense layer between two adjacent double membranes have the capacity to nitrifi. After transfer of acetate cells into yeast extract-peptone solution (3 g/l) poor growth started following 4–6 weeks of incubation. Such cells regenerated an activity of nitrite oxidation which was comparable to nitrite oxidizers which had been adapted



Figs. 4–9. Cells of *Nitrobacter agilis* grown heterotrophically. Thin sections, glutaraldehyde, OsO_4, Epon. Magnification 50000× *PP* inclusions of polyphosphate; *GL* glycogen; *DM* double membrane; *PHB* PHB-granules; *Nb*$_1$ phage-like particles Nb$_1$

Fig. 4. Cells grown with yeast extract-peptone (0.3% YP)
Fig. 5. Cells grown with α-ketoglutarate + 0.015% YP
Fig. 6. Cells grown with formate + 0.15% YP
Fig. 7. Cells grown with pyruvate + 0.15% YP
Fig. 8a and b. Cells grown with acetate + 0.015% YP
Fig. 9. Acetate cells starting nitrite oxidation after 3 weeks incubation in the presence of acetate and nitrite

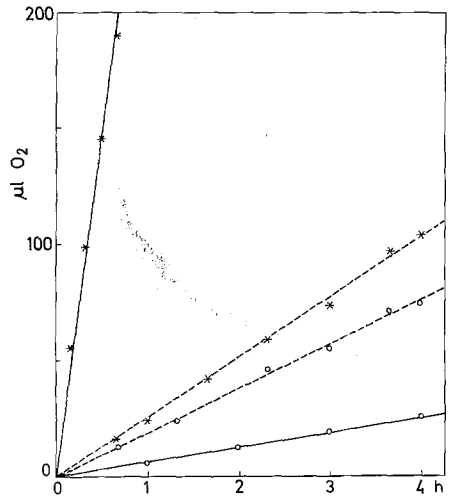


Fig. 10. Endogenous respiration and nitrite oxidation of heterotrophically grown cells of *Nitrobacter agilis*. Warburg vessel: 2 ml of bacterial suspension. Center well: 0.1 ml of KOH (10%). O——O endogenous respiration; *——* nitrite oxidation in the presence of 2 mg $NaNO_2$ of cells grown in YP after 4 transfers in acetate (447 µg N); O----O endogenous respiration; *----* nitrite oxidation in the presence of 2 mg $NaNO_2$ of cells grown the 5th transfer in acetate (428 µg N)

to heterotrophic growth in yeast extract-peptone (Table 2). The activity was 30 times that of acetate cells (Fig. 10). The results proved that nitrite oxidase can be induced by substances present in yeast extract-peptone. The nature of the inducing substances is unknown.

6. *Characterization of Chemoorganotrophic Cells*

To rule out contaminants cultures of *Nitrobacter* were tested regularly by inoculation on 5 organic media, by examination with the light microscope, and by use of the electron microscope. The following properties were characterizing chemoorganotrophic cells of *Nitrobacter agilis*. They seem to be obligate to a pure culture.

In contrast to other bacteria chemoorganotrophic growth was very slow. The shortest generation time, we determined, was 65 h with pyruvate as carbon and energy source. Therefore, after inoculation of 2.5 – 5% (v/v) on fresh medium it took 9 – 10 days before growth occurred. Turbidity after a few days is demonstrating contamination of the culture. On the other hand every chemoorganotrophic pyruvate cell of *Nitrobacter* was able to start nitrification as shown by dilution technique.

Cells were irregular in shape. As revealed by the light microscope rods and more coccoid forms were accompanied by budding and swollen cells of different size (Fig. 11). To exclude pleomorphic contaminants which have a generation time of about 70 h ultrastructural research was done. In comparison to mixotrophic bacteria (Fig. 12a and b) chemoorganotrophic bacteria (Figs. 4 – 8) had an identical structure of the cell wall.

Mixotrophic and chemoorganotrophic *Nitrobacter* cells were possessing double membranes and polyhedral particles Nb_1 as well. The storage materials were PHB, polyphosphate and glycogen-like inclusion bodies.

With exception of cyt. a_1 the difference spectra of cytochromes were in good agreement. There were distinct peaks of cyt. *c* (α-band, 550 nm), cyt. a_3 (α-band, 605 nm) but no peaks of cyt. *b* (α-band, 560 nm).

In accordance preliminary studies on the TCC enzymes of nitrite and pyruvate cells have revealed striking similarities. The levels of enzyme activities of both chemoorganotrophic and chemolithotrophic *Nitrobacter* cells were nearly identical (Steinmüller, personal communication).

DISCUSSION

The fact that only a few investigators have studied the heterotrophic growth of nitrifiers may be explained by the difficulties connected with recognition and exclusion of contaminants. Since *Nitrobacter* cells have no uniform morphology the value of microscopic control for detecting pleomorphic contaminants is restricted. Depending on growth conditions there dominate coccoid, rod-like, pear-shaped, budding and swollen cells. The classic method to check the purity of lithotrophic cultures by inoculation into yeast extract-peptone medium is, of course, not applicable to heterotrophically growing cells if contaminants are present which have a generation time about 70 h and more. Consequently, techniques like hybridization of nucleic acids, serology, and ultrastructural research have to be applied. In this study the latter method was used to check the purity of cultures.

Electron microscopic studies of thin sections revealed that the structure of the cell wall is invariable in lithotrophic and chemoorganotrophic cells. In addition, the cells from varying growth conditions possess double membranes and polyhedral particles Nb_1. Storage products as polyphosphates, PHB, and glycogen-like inclusions characteristic in Nitrobacteriaceae (Watson, 1971) were also found in cells growing chemoorganotrophically. Based on structural and biochemical conformity the heterotrophic strains in this study have to be regarded as pure bacterial cultures.

Neither theoretical considerations (Rittenberg, 1972) nor experimental facts (Coleman, 1907/1908;

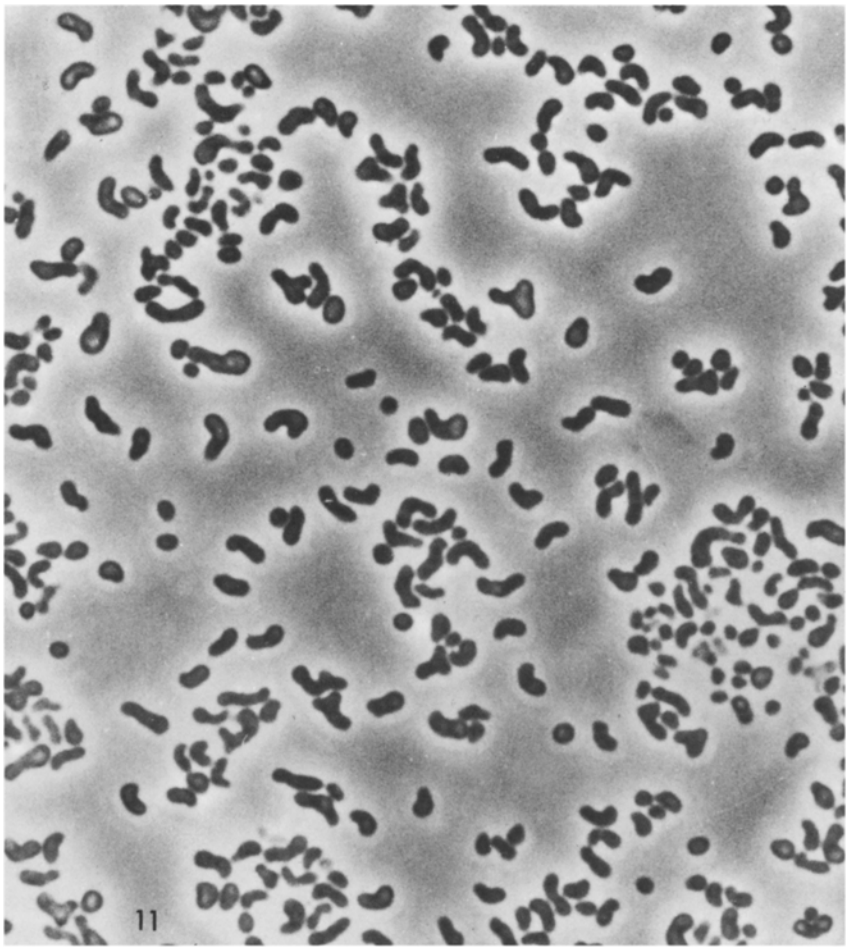


Fig. 11
Phase contrast photomicrograph of pyruvate grown cells of *Nitrobacter agilis*, to illustrate the diversity of cell form. Magnification 4000×

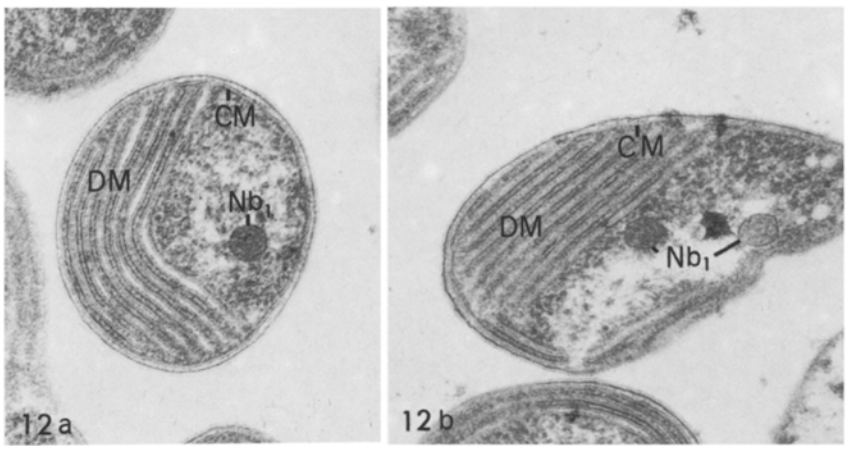


Fig. 12a and b
Mixotrophic cells of *Nitrobacter agilis* grown with nitrite + 0.15% YP. Thin sections, glutaraldehyd, OsO_4, Epon. Magnification 50000×. *DM* double membrane; *CM* cytoplasma membrane; Nb_1 phage-like particles Nb_1. (a) T.S. (b) L.S.

Delwiche and Finstein, 1965; Ida and Alexander, 1965) are in favor of the idea that nitrifying bacteria obligately depend on an inorganic energy source. Smith and Hoare (1968) demonstrated heterotrophic growth with acetate and casein hydrolysate. Their results could be confirmed in this study. Depart from general accordance the strain used in this study after growth with acetate or pyruvate in the presence of 0.015% yeast extract-peptone loses the ability of oxidizing nitrite. In the presence of nitrite or yeast extract-peptone at higher concentrations the cells regain the ability to oxidize nitrite. Being unknown whether some cells may lose the ability for lithoautotrophic growth — as known in *Ferrobacillus ferrooxidans* (Shafia et al.,

1972) – it can be said that the nitrite oxidase is inducible in most of the cells. It is intended to find out in further studies whether loss of cyt. a_1 and activity of nitrite oxidation, which seems to be parallelled by the loss of polarly arranged membranes is correlated with the lipid protein patterns of the membranes.

Pyruvate has been shown to be an effective substrate for chemoorganotrophic growth. When comparing substrate respiration of *Nitrobacter* and ordinary heterotrophic bacteria it is striking that in the presence of pyruvate O_2 uptake is 2–3 times that of endogenous respiration in *Nitrobacter*; in *Azotobacter vinelandii* it is 8–10 times that of endogenous respiration (Knowles and Smith, 1970). Because of the good growth of *Nitrobacter* in the presence of pyruvate the assumption is made that oxidative phosphorylation is highly efficient. The P/O ratio is supposed to be better than P/O = 1 in nitrite oxidation (Aleem, 1968).

A substrate supplied with pyruvate as carbon and energy source and yeast extract-peptone as nitrogen and vitamin source probably is also suitable for chemoorganotrophic growth of other nitrifiers. As in thiobacilli (Andersen and Lundgren, 1969; Johnson and Abraham, 1969; Matin and Rittenberg, 1970, 1971) there have to be expected transitional stages between obligate and facultative lithoautotrophy. The ubiquitous existence of nitrifiers in nature possibly is not to be explained by the presence of the specific inorganic electron donors at any place and any time but rather by the ability to utilize different organic compounds for heterotrophic growth. As a rule the bacteria preserve the capability of nitrification when growing heterotrophically.

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