Nutrition and Physiology of *Nitrobacter agilis*[1]

M. I. H. Aleem[2] and M. Alexander

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York

Nitrate formation in nature is one of the more important phases of the nitrogen cycle, yet the number of species capable of forming this anion are quite restricted. In addition to the chemoautotrophic bacteria, only *Aspergillus flavus* and certain other fungal strains have been verified to be capable of nitrate formation (Eylar and Schmidt, 1959). The information presently available thus indicates that this critical portion of the sequence of biochemical transformations of nitrogen is catalyzed only by a few unique microorganisms.

The dominant genus in the second step of nitrification is *Nitrobacter*, but only two species are currently recognized, *Nitrobacter winogradskyi* and *Nitrobacter agilis* (Breed, Murray, and Smith, 1957). Strains have been obtained from many diverse environments, but the areas of dissimilarity have not been sufficiently great to warrant the designation of new species. Since the nitrite-oxidizing bacteria are of great economic importance in agriculture and in circumstances where their activities are preferentially inhibited so that nitrite accumulates (Stojanovic and Alexander, 1958), a study was initiated to ascertain certain of the nutritional and physiological properties of the less investigated species in the genus, *N. agilis*.

Knowledge of the mineral nutrition of the nitrite oxidizing autotrophs is incomplete and often ambiguous. Thus, the optimum iron concentration for growth of *N. winogradskyi* is reported to be about 6 ppm for the oxidation of 200 ppm nitrite-nitrogen (Meiklejohn, 1953), but application of the energy efficiency values of this organism, of the order of 100 parts nitrogen oxidized to 1 part carbon assimilated (Meyerhof, 1916a), demonstrates that this iron level exceeds the amount of cell carbon formed. Specific requirements for magnesium, phosphate, and nitrite have also been reported (Bömeke, 1950; Meiklejohn, 1952; Meyerhof, 1916a), but the cultural conditions were far from adequate.

Experimental Methods

Nitrobacter agilis was grown in 500-ml flasks containing 200 ml of the following medium: 0.3 g KNO_2; 0.175 g K_2HPO_4; 0.175 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 1.5 g $KHCO_3$; and 35 μg $FeSO_4 \cdot 7H_2O$ in 1.0 L deionized, distilled water. Each flask received 5 ml of a 24 to 48 hr inoculum and was incubated on a 285-rpm rotary shaker at 30 C.

To study its inorganic nutrition, the organism was grown in media from which the nutrient under study was omitted for three consecutive subcultures with an incubation period of 4 days per subculture. For each transfer, 0.5 ml inoculum was added to the culture flask containing 200 ml medium. The requirements for each nutrient were studied by omitting the nutrient from the medium described and adding graded amounts to flasks in a standard series. Aliquots of 1 ml were withdrawn at regular intervals for nitrite analysis, and fresh nitrite additions were made after the initial amount was metabolized until a point was reached where no further oxidation occurred with subsequent additions. The medium was purified essentially by the alumina method of Donald *et al.* (1952). All glassware was acid washed prior to use, and the water included in the culture vessel was prepared by passing steam-distilled water through cation and anion exchange resins.

Nitrite was determined by the alpha-naphthylamine-sulfanilic acid procedure (American Public Health Association, 1955) and nitrate by the method of Buckett *et al.* (1955). Since nitrite interferes in the determination of nitrate, it was eliminated by heating at 65 to 70 C for 10 min in the presence of 8 per cent oxalic acid and 10 per cent urea. The determination of effective aeration was made by the sulfite oxidation method (Cooper, Fernstrom and Miller, 1944) and results expressed in terms of mM O_2 per L per min.

The bacterium was cultivated and cell extracts prepared from the resulting cell paste by the methods described in a previous paper (Aleem and Alexander, 1958). When the cultures were growing exponentially, the ratio of nitrobacter numbers to nitrite-nitrogen was of the order of 30,000:45,000 cells for 1.0 μg nitrite-N oxidized. For manometric investigations, each reaction vessel contained 20 μM nitrite, 5 μM $MgSO_4$ and 50 μM phosphate buffer at pH 8.0 in the main compartment, 0.2 ml of 20 per cent KOH in the center well and cell suspension in the side arm. Oxygen consumption was measured at 30 C.

Experimental Results

Although nitrite itself is a bacteriostatic agent, the organism can obtain all of its energy from the oxi-

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[2] Present address: McCollum Pratt Institute, The Johns Hopkins University, Baltimore, Maryland.

dation of this anion. As little as 130 ppm nitrite-N were found to prolong the lag phase of growth to 2 to 3 days whereas growth did not occur in media initially containing 1100 ppm. No detectable lag period was observed in cultures grown in solutions with initial concentrations of less than about 40 ppm nitrite-nitrogen. Once the organism was developing exponentially, additions of up to 500 ppm of nitrite-nitrogen could be made without suppressing the growth, and the logarithmic phase lasted until about 1500 ppm nitrogen were oxidized. The maximum nitrite transformed under these conditions was found to be *ca* 4000 ppm nitrogen, and the minimum generation time observed was about 7 hr.

Little nitrite was oxidized in media containing less than 0.05 ppm added phosphorus whereas higher levels increased the total oxidation to about 3000 ppm nitrogen (table 1). Approximately 5 ppm phosphorus seems to be the minimal level required to support maximal nitrite conversion. There was no nitrification in the absence of magnesium, the bacterial response paralleling the quantity added up to about 5 ppm. Nitrite oxidation was also enhanced by the addition of iron although the solution was never sufficiently iron deficient since growth in the purified medium was one-third of that in the fully supplemented solution. The amount of nitrite oxidized was doubled by 0.001 and trebled by 0.007 ppm Fe when compared with controls receiving none whereas higher concentrations were without effect. The addition of 0.01 ppm or more copper or manganese to the complete medium failed to increase the rate or total amount of nitrate produced.

To determine the aeration rate necessary for *N. agilis*, different levels of effective aeration were attained by the use of shaken and nonshaken cultures and by varying the amount of liquid in each vessel. An effective aeration rate of 0.038 mM O_2 per L per min was found to permit rapid oxidation and was adequate to support the population that developed when the energy supply is of the order of 35 ppm nitrite nitrogen. Increasing the aeration to 0.30 mM O_2 per L per min did not significantly alter the growth pattern. When O_2 was limiting, levels of 0.018 mM O_2 per L per min being inadequate for rapid proliferation, multiplication was retarded and was apparently governed by oxygen diffusion. It is interesting that excessive aeration (1.43 mM O_2 per L per min) prolonged the lag period although the subsequent nitrification proceeded readily.

If the effect of hydrogen ion upon the bacterium is measured by determining the O_2 consumed with nitrite as substrate, it is noted that *N. agilis* can respire within a wide pH range, from 5.5 to 9.8 (figure 1). The results demonstrate that alkaline conditions are optimal for the bacterium, and the greatest oxygen uptake was recorded at pH 8.0 although the respiratory rate was still high at pH 7.0 and 8.5.

The rate of nitrite oxidation was not affected by the presence of 500 ppm nitrate-nitrogen initially in the culture fluid, but concentrations of 1000 to 2000 ppm prolonged the lag period whereas no nitrification occurred if 5000 ppm nitrate-nitrogen were present at the

TABLE 1

Response of Nitrobacter agilis to phosphorus, magnesium, and iron

Nutrient Addition*	Nitrite-N Oxidation		
	P	Mg	Fe
ppm	*ppm*	*ppm*	*ppm*
0.0	136	3	1050
0.001			2150
0.003			2350
0.005	164		
0.007			3050
0.025		197	
0.05	958		2800
0.20		464	2800
0.50	2140		
1.0		1591	
5.0	2920	2405	
25.0	2975	2341	

* Rates of addition are in terms of the element. Incubation of 30 days for P and Mg and 40 days for Fe.

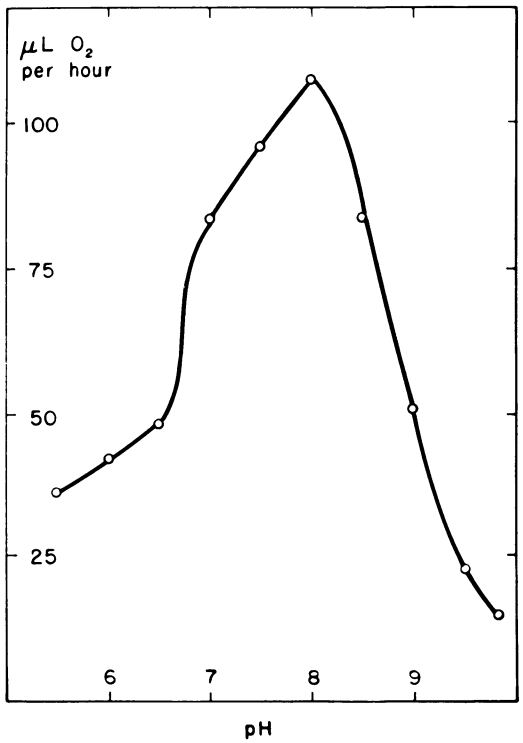

Figure 1. Influence of pH on the oxidation of nitrite. The manometer vessel contained 0.3 ml cell suspension, 5 μM $MgSO_4$, 10 μM KNO_2 and 50 μM buffer. Phosphate was the buffer below pH 8.0, tris(hydroxymethyl)aminomethane at 8.0 to 9.0 and carbonate above pH 9.0.

time of inoculation. The addition of 2000 and 5000 ppm to active cultures, however, did not lead to a significant depression. Thus, it is apparent that the accumulation of nitrate does not interfere with the process of nitrite oxidation by these microorganisms.

To assess the need for surface adsorption for development of these chemoautotrophic nitrifying bacteria, growth of *N. agilis* in clear media and in solutions supplemented with 1.0 per cent $CaCO_3$ were compared. The organisms grew with no detectable response to the presence of the particulate substances. These results are in agreement with data reported for the bacteria catalyzing the first step in nitrification (Engel and Alexander, 1958).

In spite of the strictly chemoautotrophic nutrition of the nitrifying bacteria, various organic substances have been reported to have a favorable effect suggesting thereby a possible nutritional inadequacy. Experiments were performed to study the influence of a number of vitamins and amino acids on *N. agilis*. The results recorded in table 2 show that thiamine, pyridoxine, riboflavin and biotin had no effect on oxygen uptake with nitrite as substrate. Similarly, glutamic acid, histidine, threonine, valine, isoleucine, lysine, methionine, phenylalanine, tryptophane and arginine at concentrations of 30 to 400 μg per ml and casamino acids at a concentration of 1.7 mg per ml did not stimulate oxygen consumption.

Although the natural substrate for soil nitrification is ammonium, laboratory and field data has shown that nitrite accumulates in alkaline soils treated with ammonium salts or ammonium-forming materials. The effect of ammonium as a potential inhibitor was investigated by including it in media inoculated with *N. agilis*. As little as 10 ppm ammonium-N suppressed the bacterium. For example, cultures without ammonium completely oxidized 45 ppm nitrogen in 4 days whereas 10 days were required to nitrify the same amount in the presence of the ammonium salt. The inhibition was more pronounced in the presence of 20 ppm of ammonium, and the reaction required 17 days. At 50 ppm NH_4-N, the oxidation proceeded very slowly and only 40 per cent of the substrate was transformed by the 17th day after which no further conversion took place. Concentrations of 100 ppm ammonium-N completely prevented growth initiation.

The toxicity of various concentrations of ammonium upon oxygen uptake was determined at various pH levels by use of nonproliferating cell suspensions. A pronounced inhibition was noted at pH 9.5 (figure 2) with as little as 0.3 μM suppressing the transformation. Greater concentrations of the NH_4 produced a more marked depression, the rate being reduced 28, 57, and 70 per cent by 0.3, 1.0, and 3.0 μM per reaction vessel. Respiration was not affected at pH 6.0 and 6.5 nor at pH 7.0 with 5 μM ammonium per reaction vessel, and concentrations of 10 μM depressed oxygen uptake at the latter pH by only 9 per cent (table 3). At pH 7.5, the toxicity was greater and the inhibition became magnified with increasing alkalinity and greater inhibitor concentration. The suppression was most pronounced at pH 9.5 with an 82 per cent inhibition in oxygen uptake.

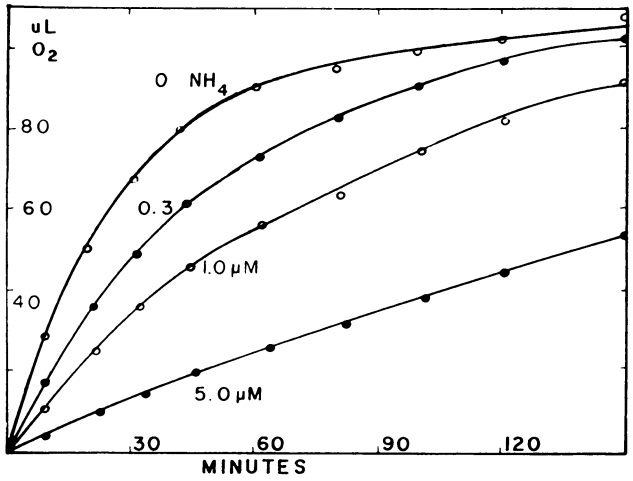

Figure 2. Inhibition of nitrite oxidation at pH 9.5 by ammonium sulfate.

TABLE 2

Effect of vitamins on the oxygen uptake by Nitrobacter agilis

Treatments	Vitamin Concentration	O_2 Uptake
	μg/flask	μL/hr
Control	—	216
Thiamine	50	222
Pyridoxine	50	213
Riboflavin	50	210
Biotin	50	204

Each Warburg flask contained 0.3 ml of a cell suspension equivalent to 0.211 mg nitrogen. The other reactants in the vessel are: 50 μM phosphate, 20 μM nitrite and 5 μM of magnesium sulfate.

TABLE 3

The effect of ammonium at different pH levels to Nitrobacter agilis

pH	Inhibition at Different Ammonium Concentrations	
	5 μM	10 μM
	%	%
6.0	0	0
6.5	0	0
7.0	0	9.0
7.5	17	17
8.0	19	30
8.5	28	41
9.5	82	82

The reaction vessel contained 0.3 ml cell suspension equivalent to 0.221 mg N. Other reactants as in figure 1.

The toxicity is not concerned with the nitrite-oxidizing enzyme *per se* since ammonium added to cell extracts incubated at pH 9.5 did not reduce the formation of nitrate from nitrite. This was determined by incubating 0.5 ml extract containing 0.28 mg cell nitrogen with 5 to 50 μM of ammonium in a system containing 58 ppm nitrite-N, 100 μM phosphate, 5 μM ferric iron in a volume of 5 ml. The lack of an ammonium inhibition to the nitrite-activating enzyme system is in striking contrast to its influence upon the intact organism.

Discussion

The rate of nitrite oxidation by *N. agilis* is not significantly affected by substrate levels up to 400 ppm nitrogen, but a lag is present where the oxidizable compound is present in large amounts. Once the culture is in the logarithmic phase, it will tolerate high nitrite with no influence on the rate of proliferation. The effect of nitrite is thus one of retarding the initiation of growth, results similar to those obtained in soil (Stojanovic and Alexander, 1958). Using a technique of successive addition, the magnitude of the autotrophic nitrate formation can be increased to very high levels.

The results demonstrate that the optimal nutrient level is approximately 5 ppm for both phosphorus and magnesium whereas the iron requirement seems to be about 0.005 ppm. However, the iron content of the purified medium was still adequate for appreciable nitrification so that the optimum level is probably somewhat higher. These data are in contrast to the findings of Meiklejohn (1953) who reports an iron requirement of the same order of magnitude as the P and K levels. The data of Meiklejohn likely result from reactions with the $CaCO_3$ present which make most of the iron unavilable to the microorganisms. Zavarzin (1958) has found a beneficial effect of molybdenum on *Nitrobacter* sp. postulating from this that a molybdoflavoprotein is concerned in the energy yielding reaction of these autotrophs. Similar findings have been obtained with *N. agilis*.

Neither B vitamins nor amino acids stimulated the organism at the concentrations used. This is further evidence in support of their strictly autotrophic nutrition. Although stimulation of growth by different organic substances has been reported, it seems likely that such observations may be the result of nonspecific physical or chemical effects in suboptimal conditions rather than a physiological stimulation. Lack of stimulation by amino acids has also been demonstrated for a *Nitrosomonas* sp. (Gundersen, 1955).

The phenomenon of ammonia toxicity was first investigated intensively by Meyerhof (1916b) who demonstrated a marked inhibition of a *Nitrobacter* strain, presumably *N. winogradskyi*, by ammonium salts and a number of aliphatic and aromatic amines. Similar suppressive influences have been reported for other *Nitrobacter* isolates (Boullanger and Massol, 1903; Bömeke, 1950) so that the action of ammonium salts in alkaline environments seems to be a generic property which is clearly of great ecological significance.

In soil, the rate of ammonium oxidation is largely independent of its concentration over wide ranges, but nitrite accumulation becomes more pronounced with greater ammonium applications suggesting that the toxic effect of ammonium is upon the second step of nitrification, that catalyzed by *Nitrobacter* species (Stojanovic and Alexander, 1958). The present work demonstrates that *N. agilis* is indeed highly sensitive *in vitro* to low concentrations of ammonium. In soil, more ammonium is required to depress the nitrite oxidation than in pure culture of the bacterium, a fact resulting at least in part from chemical adsorption of the applied nitrogen. The observed relation of pH to toxicity indicates that the two factors involved in the nitrite accumulation during the nitrification are alkalinity and substrate concentration. Since the pattern of inhibition closely parallels the shift in the ammonium-ammonia equilibrium towards the latter compound, it is likely that free ammonia is the active principle.

Summary

A study has been made of the nutrient requirements for growth of *Nitrobacter agilis*. Nitrification by the bacterium proceeds readily only when nitrite is supplied at low levels initially, but high substrate concentrations can be added to exponentially growing cultures without significant toxic effects. The end product of growth, nitrate, is likewise without influence upon actively proliferating cultures. The requirement for phosphorus, magnesium, and iron have been established.

Ammonium salts are selective inhibitors of nitrite oxidation by intact cells of *N. agilis*, the effect being apparent only in alkaline solution. The toxicity decreases with increasing hydrogen ion concentration. The oxidation by cell extracts was not retarded under similar conditions.

REFERENCES

Aleem, M. I. H. and Alexander, M. 1958 Cell-free nitrification by *Nitrobacter*. J. Bacteriol., **76,** 510–514.

American Public Health Association 1955 *Standard methods for the examination of water, sewage, and industrial wastes.* 10th ed., New York, New York.

Bömeke, H. 1950 Über die Ernährungs- und Wachstumfaktoren der Nitrifikationsbakterien. Arch. Mikrobiol., **14,** 63–98.

Boullanger, E. and Massol, L. 1903 Etudes sur les microbes nitrificateurs. Ann. Inst. Pasteur, **17,** 492–515.

Breed, R. S., Murray, E. G. D., and Smith, N. R. 1957 *Bergey's manual of determinative bacteriology.* 7th ed., Williams and Wilkins, Baltimore, Maryland.

Buckett, J., Duffield, W. D., and Milton, R. F. 1955 The

determination of nitrate and nitrite in soil. Analyst, **80,** 141–145.

COOPER, C. M., FERNSTROM, G. A., AND MILLER, S. A. 1944 Performance of agitated gas-liquid contactors. Ind. Eng. Chem., **36,** 504–509.

DONALD, C., PASSEY, B. I., AND SWABY, R. J. 1952 Bioassay of available trace metals from Australian soils. Australian J. Agr. Research, **3,** 305–325.

ENGEL, M. S. AND ALEXANDER, M. 1958 Culture of *Nitrosomonas europaea* in media free of insoluble constituents. Nature, **181,** 136.

EYLAR, O. R., JR. AND SCHMIDT, E. L. 1959 A survey of heterotrophic micro-organisms from soil for ability to form nitrite and nitrate. J. Gen. Microbiol., **20,** 473–481.

GUNDERSEN, K. 1955 Effects of B-vitamins and amino-acids on nitrification. Physiol. Plantarum, **8,** 136–141.

MEIKLEJOHN, J. 1952 Minimum phosphate and magnesium requirements of nitrifying bacteria. Nature, **170,** 1131.

MEIKLEJOHN, J. 1953 Iron and the nitrifying bacteria. J. Gen. Microbiol., **8,** 58–65.

MEYERHOF, O. 1916a Untersuchungen über den Atmungsvorgang nitrifiizierender Bakterien. I. Die Atmung des Nitratbildners. Arch. ges. Physiol., **164,** 353–427.

MEYERHOF, O. 1916b Untersuchungen über den Atmungsvorgang nitrifizierender Bakterien. II. Beeinflussungen der Atmung des Nitratbildners durch chemische Substanzen. Arch. ges. Physiol., **165,** 229–284.

STOJANOVIC, B. J. AND ALEXANDER, M. 1958 Effect of inorganic nitrogen on nitrification. Soil Sci., **86,** 208–215.

ZAVARZIN, G. A. 1958 The effect of heavy metals on nitrification (In Russian). Mikrobiologiya, **27,** 542–546.

Separation of Microorganisms by Flotation

I. Development and Evaluation of Assay Procedures[1]

A. M. GAUDIN,[2] A. L. MULAR,[3] AND R. F. O'CONNOR[3]

Department of Metallurgy, Massachusetts Institute of Technology, Cambridge, Massachusetts

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The separation of bacterial cells from the growth menstruum is a routine operation which continues to demand more than its fair share of effort from workers in such fields as physiology, immunology and cytology. Preparation of "clean" cell suspensions by centrifugation is arduous and yields are often low.

The observation that masses of cells collected in the foam above the liquid level in fermentors led Boyles and Lincoln (1958) to use vigorous aeration of the culture medium after growth as a means for removing and concentrating spores of *Bacillus anthracis*. Quantitative estimates of the degree of separation and/or concentration were based on viable counts. Success in separating spores of *Bacillus cereus* T (formerly called *Bacillus cereus* var. *terminalis*) from autolyzed culture by frothing was reported by Black *et al.*, (1958).

The concept of the separation of particles in liquid by foaming has long been known in the field of mineral engineering, and is the basis for "flotation" processes. Flotation studies on minerals have provided, in addition to rapid, efficient separation techniques, much basic information concerning the structures and surface configurations of mineral particles. Flotation testing of *Bacillus subtilis* var. *niger* was undertaken because of the possibility that such investigation might lead to the development of methods for separating and concentrating bacterial cells, and might also provide a new tool for studying the composition and molecular orientation of cell surfaces.

This paper presents the results of some initial flotation testing with *B. subtilis* var. *niger* related to the development of two assay techniques for the evaluation of flotation tests. One assay is based upon a chemical analysis for a constituent of spores, pyridine-2,6-dicarboxylic acid (dipicolinic acid); and the other assay is based upon a procedure in which cells, spores, and other particles are visually counted.

DESCRIPTION AND DISCUSSION OF FLOTATION

Definitions. Flotation may be defined as a process for separating finely divided solids from each other (Gaudin, 1957). The solids are suspended in water through which gas bubbles are caused to flow. Separation takes place when particles of one type adhere to gas bubbles and are carried to the top of the liquid as a froth, whereas particles of other types adhere to the liquid and remain in suspension.

That product of a flotation operation which contains the valuable or preferred constituent is called the "concentrate" (C) and usually is the froth or "float." The other product contains the worthless constituent and is called the "tailing" (T) which usually is the non-

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[2] Professor of Mineral Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts.

[3] Staff member, Division of Sponsored Research, Massachusetts Institute of Technology, Cambridge, Massachusetts.