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Autotrophy in *Nitrosocystis oceanus*[1]

PETER J. LEB. WILLIAMS[2] AND STANLEY W. WATSON
Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

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Enzymatic assays of cell-free extracts of the ammonia-oxidizing bacterium *Nitrosocystis oceanus* failed to establish that the biochemical basis of its obligate autotrophy stemmed solely from a metabolic defect. All of the Embden-Meyerhof enzymes except phosphofructokinase, and all of the tricarboxylic acid-cycle enzymes, as well as reduced nicotinamide adenine dinucleotide oxidase, were found in these extracts. A phosphoenolpyruvate-CO^2-fixing system was also demonstrated. Resting cells incubated with ^{14}C-D-glucose and ^{14}C-L-glutamate and cells grown in the presence of ^{14}C-labeled glucose, glutamate, pyruvate, and methionine incorporated these compounds into cellular material, but at a level too low to provide the cells' major carbon and energy needs.

The bacteria usually considered as obligate chemoautotrophs include some of the thiobacilli and all of the nitrifying bacteria. Whether a common basis for obligate autotrophy exists in these bacteria has not been established. Both permeability barriers and metabolic defects have been considered as factors which may prevent these organisms from growing heterotrophically.

For many years after Winogradsky's (38) discovery of chemoautotrophic bacteria, it was accepted that they used only inorganic compound and carbon dioxide as sole energy and carbon sources. Recent studies have established that all of the obligate chemoautotrophs thus far examined can incorporate exogenously supplied organic compounds into cellular material (5-7, 12, 14, 27-29). Yet it is still believed that *Thiobacillus thiooxidans*, *T. thioparus*, *T. neopolitanus*, and all the nitrifying bacteria, with the possible exception of *Nitrobacter agilis* (27, 28), cannot use organic compounds as sole energy and carbon sources.

The work of Smith et al. (29) and Kelly (14) indicates that the failure of the thiobacilli to live heterotrophically may stem from metabolic defects. Smith et al. found that two species of thiobacilli, *T. thiooxidans* and *T. thioparus*, lacked α-ketoglutarate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) oxidase and had unusually low levels of malate and succinate dehydrogenase. Of the above defects, Smith et al. considered the lack of NADH oxidase the most serious obstacle to heterotrophic growth. As these authors pointed out, without NADH oxidase, the only way an obligate autotroph could conceivably generate adenosine triphosphate (ATP) by the oxidation of organic compounds would be by the "substrate level phosphorylations accompanying glycolysis, a process that might support growth if they could mediate uncoupled transfer of electrons to oxygen (for example, via flavoprotein enzymes)." However, all other investigators failed to determine whether obligate autotrophs had all the glycolytic enzymes to provide energy via this metabolic pathway.

Even among the thiobacilli, there does not seem to be a common biochemical basis for obligate autotrophy, for Trudinger et al. (32) found that *T. neapolitanus* had NADH oxidase. Kelly (14) suggested that the lack of intermediates of the Krebs cycle between α-ketoglutarate and oxalacetate in *T. neapolitanus* was primarily responsible for the failure of this organism to live heterotrophically.

No similar explanations exist to account for the autotrophic nature of the nitrifying bacteria. In fact, some doubt exists as to whether all nitrifying bacteria are obligate autotrophs, since Smith and Hoare (27) found that *N. agilis* could be cultured heterotrophically. In the present investigation, we worked with another nitrifying bacterium, *Nitrosocystis oceanus* (36; S. W. Watson, Abstr. 8th Intern. Congr. Microbiol., Montreal, 1962), which has not been grown heterotrophically. The aim of this study was to determine whether metabolic defects or permeability barriers prevent this organism from using four organic compounds as major carbon or energy sources.

[1] Contribution no. 2150 from the Woods Hole Oceanographic Institution, Woods Hole, Mass. 02543.

[2] Present address: Department of Oceanography, The University, Southampton, England.

MATERIALS AND METHODS

Biological material. Pure cultures of *N. oceanus* strain 107 (36), originally isolated from open Atlantic waters, were used in all experiments. This organism is a coccoid marine nitrifying bacterium, which oxidizes ammonia to nitrite and is characterized by possessing a complex cytomembrane system (22, 24, 36).

N. oceanus was grown in *p*H-stat fermentors on a semicontinuous-flow basis with the mineral salts medium of Watson (36). Rigid precautions insured that cultures remained free from contaminants. Cultures were checked visually and culturally for contaminants, daily, before and after harvesting, and during the course of experiments.

For visual examination, cells were concentrated by centrifugation and examined by means of a phase-contrast microscope. *N. oceanus* was readily distinguished from most possible contaminants because of its unique morphology (36).

For cultural determination of purity, 1 ml of the culture was inoculated into a sterile medium consisting of 15 ml of seawater enriched either with 0.02% yeast extract plus 0.05% peptone (Difco) or one-quarter-strength Nutrient Broth (Difco).

Some contaminants detected by visual examination failed to grow in the above organic media and in a variety of other organic media used by Watson (36) in previous experiments. Consequently, visual as well as cultural checks were made to assess purity.

Cells for enzyme assays were harvested at 20,000 $\times$ *g* in a refrigerated centrifuge at 5 C from cultures, in the middle of the exponential growth phase, having a 0.02 M nitrite concentration. The cell yield was approximately 100 mg of wet cells per liter of culture. After harvesting, the cells were washed in sterile seawater (four to six times) until the final nitrite concentration was less than 2 μM.

After washing, 2 to 3 g of wet cells (144 mg of protein per g of wet cells) were resuspended in 10 ml of 0.02 M phosphate buffer or tris(hydroxymethyl)-aminomethane(Tris) buffer (*p*H 7.8) and were disrupted with a French pressure cell (Aminco) at 16,000 lb/in^2. The ruptured cells were centrifuged at 20,000 $\times$ *g* at 5 C for 20 min to remove whole cells, and the supernatant fluid was used for enzyme assays.

The protein content of the above extracts was determined by the method of Lowry et al. (20). A solution of bovine serum albumin was used as a protein standard.

Oxygen consumption. Oxygen consumption was measured by means of an oxygen electrode (13) and recorded with a Leeds and Northrup recorder operated at 1 mv. Full-scale deflection at 1 mv was equivalent to 1.0 μmole of oxygen per liter.

Assay of enzymes. Cell-free extracts of *N. oceanus* were assayed for enzyme activity within 6 hr after the harvesting of whole cells. A Gilford model 2000 recording spectrophotometer was used for all spectrophotometric determinations. All reactions were run at room temperature (21 to 23 C).

Most enzymes were assayed by published methods; these are as follows: hexokinase (37); glucose-6-phosphate dehydrogenase (15); 6-phosphogluconate dehydrogenase (11); glucose-6-phosphate isomerase (26); phosphofructokinase (19, 34); glyceraldehyde-3-phosphate dehydrogenase (35); the overall activities of phosphoglycerate phosphomutase, phosphopyruvate hydrase (enolase), and pyruvate kinase (16); citrate and malate synthetase (8); fumarate and aconitate hydratase (23); isocitrate lyase (8); α-ketogluterate dehydrogenase (9, 10, 25); NADH oxidase (29); fructose diphosphate aldolase (4); and pyruvate kinase (16).

Glycerokinase and glycerophosphate dehydrogenase were measured by a method based on an enzymatic determination of glycerol (1). Isocitrate dehydrogenase was measured by following nicotinamide adenine trinucleotide (NADP) reduction, malate dehydrogenase by following nicotinamide adenine dinucleotide (NAD) and NADP reduction, and succinate dehydrogenase by following the reduction of mammalian cytochrome *c*. Acetyl coenzyme A (CoA) was prepared by acetylating CoA with acetic anhydride by the method of Stadtman (30).

Carbon dioxide fixation by cell-free extracts. The extract with various reactants in a final volume of 1 ml was incubated at room temperature (21 to 23 C); after 15 min, the reaction was stopped by the addition of 100 μliters of 1.0 N HCl. This reaction mixture was aspirated to remove all traces of radioactive carbon dioxide. Samples (100 μliters) were placed on planchettes as 100 or so discrete spots, and the radioactivity was measured with a gas-flow detector (model D-47; Nuclear-Chicago Corp., Des Plaines, Ill.). The radioactive products of the reaction were examined by two-way paper chromatography and subsequently by radioautography. The solvent systems were those described by Kornberg (17).

Incorporation of ^{14}C-labeled organic compounds by resting cells. Wet washed cells (10 mg), grown in a *p*H-stat fermentor as previously described, were resuspended in 500 μliters of sterile seawater containing 1 μmole of ammonium ion and either uniformly labeled ^{14}C-D-glucose or L-glutamate (1.0 μc, 0.2 μmoles). At intervals, 100-μliter samples were withdrawn and placed on membrane filter discs (HA; Millipore Corp., Bedford, Mass.; 0.45 μm pore size). The filter was washed 10 times with sterile seawater, placed on planchettes, and counted.

Incorporation of ^{14}C-labeled organic compounds by growing cells. Cells were grown in 1-liter fermentors equipped with automatic *p*H control. At the start of the experiment, these fermentors were inoculated with 10^8 cells, and growth was allowed to continue until there were 10^{10} cells. Immediately after inoculation, uniformly labeled-^{14}C glucose or glutamate (10 μc, 10 μmoles), pyruvate-3-^{14}C (10 μc, 10 μmoles), or ^{14}C-(methyl) methionine (2 μc, 0.1 μmoles) was added to the inorganic growth medium of Watson (36). Prior to addition, all organic compounds were sterilized by filtration.

Cells were harvested by centrifugation at 20,000 $\times$ *g* for 20 min at 5 C and subsequently washed five to eight times in sterile seawater. The yield of cells was approximately 100 mg, wet weight (14 mg of protein).

Fractionation of cellular material. The soluble cell contents were extracted by suspending washed cells in 10% trichloroacetic acid at room temperature and centrifuging the suspension for 20 min at 33,000 × g. Trichloroacetic acid was removed from the supernatant fluid by five ether extractions. This solution was made alkaline, evaporated to dryness, resuspended in a small volume of water, placed on chromatography paper, and developed as described below.

The trichloroacetic acid-insoluble fraction, containing the cellular proteins, was washed five times in 1% trichloroacetic acid containing 1% of the equivalent unlabeled material to remove any adsorbed radioactivity. The radioactivity present in the trichloroacetic acid-insoluble fraction was determined by homogenizing the precipitate and plating out 100-μliter samples.

The remaining precipitate was washed five times in 1% trichloroacetic acid and once in distilled water; it was then hydrolyzed for 16 hr with 6 N HCl at 105 C. The hydrolysate was freed from HCl by repeated evaporation to dryness under reduced pressure, made alkaline, evaporated, and placed on chromatography paper.

The amino acids were separated by two-way ascending chromatography in the solvent system of Benson et al. (1). Radioactive compounds on the chromatograms were located by radioautography with Kodak "No Screen" x-ray plates (Eastman Kodak Co., Rochester, N.Y.). The appropriate areas on the chromatogram were cut into small strips and eluted by shaking in a known volume of distilled water.

Determination of the specific radioactivity of the eluted material. The radioactivity of the eluate was measured by pipetting out 100-μliter samples on planchettes. Their absolute radioactivity was measured with a counter previously calibrated with a standard ^{14}C source.

The amino acid content of the eluate was measured by the ninhydrin method of Matheson and Tattrie (21). Since ammonia interferes with the assay, the eluate was raised to *p*H 10, alternately evaporated to dryness, and redissolved four times. After the fourth evaporation, the eluate was returned to its original volume. Prior to the addition of the ninhydrin reagents, the *p*H was adjusted to 5. Salts reduce the sensitivity of the test; therefore, appropriate controls were run and corrections were made.

Radioactive materials. All radioactive materials were purchased from New England Nuclear Corp., Boston, Mass. Glucose and glutamic acid were purified prior to use by two-way paper chromatography in the solvent system of Benson et al. (1).

RESULTS AND DISCUSSION

Enzymes of the Embden-Meyerhof pathway. All of the enzymes of the Embden-Meyerhof sequence were detected in cell-free extracts of *N. oceanus*, with the exceptions of phosphofructokinase (Table 1) and triosephosphate isomerase. Since the latter enzyme was found in this organism by Campbell et al. (2), we did not attempt to demonstrate its presence in this investigation. Although several published methods were used (19, 34), phosphofructokinase activity was not detected.

TABLE 1. *Specific activities of the enzymes of the Embden-Meyerhof pathway, pentose phosphate pathway, and glycerol metabolism in cell-free extracts of N. oceanus*

Enzyme	Specific activity[a]
Hexokinase	0.054
Glucose 6-phosphate isomerase	0.10
Phosphofructokinase	0.00
Fructose diphosphate aldolase	0.23
Glyceraldehyde-3-phosphate dehydrogenase	0.018
Phosphoglycerate phosphomutase, phosphopyruvate hydrase and pyruvate kinase (overall activity)	0.045
Pyruvate kinase	0.050
Glycerokinase	0.00
Glycerophosphate dehydrogenase	0.00
Glucose 6-phosphate dehydrogenase	0.0076
6-phosphogluconate dehydrogenase	0.0018

[a] Expressed as micromoles of substrate oxidized per minute per milligram of protein.

Enzymes of the glycerol metabolism. Neither of the two enzymes required to facilitate the entrance of glycerol into the Embden-Meyerhof pathway were found in cell-free extracts (Table 1). Their absence probably did not result from an inhibitor, because added glycerokinase and glycerophosphate dehydrogenase retained their activities in the presence of the extract.

Activities of the tricarboxylic acid cycle enzymes. All of the enzymes of the tricarboxylic acid cycle were detected in cell-free extracts of *N. oceanus*. The specific activities of these enzymes are listed and compared with those reported in other autotrophs (Table 2). In *N. oceanus*, the specific activities of these enzymes are similar to those found in other obligate and facultative autotrophs, with the exception of succinate dehydrogenase. It is significant that the specific activity of succinate dehydrogenase in *N. oceanus* is two orders of magnitude lower than in the facultative autotrophs. Similarly, the specific activity of succinate dehydrogenase is low in *N. agilis* and *T. thioparus*, which are usually considered obligate autotrophs.

Since *N. oceanus* and *N. agilis* have NADH oxidase and all the enzymes of the tricarboxylic acid cycle, they have the metabolic potential to use organic molecules as sole energy and carbon sources. However, the low specific activity of

TABLE 2. *Specific activities of enzymes of the tricarboxylic and glyoxylic acid cycles in cell-free extracts of N. oceanus and other obligate and facultative autotrophs*

Enzyme	Specific activity[a]					
	N. oceanus	*Hydrogenomonas* sp.[b]	*Hydrogenomonas* sp.[c]	*T. thioparus*[d]	*T. intermedius*[e]	*N. agilis*[f]
Citrate synthase	0.013	0.033	0.036			0.016
Aconitate hydratase	0.25	0.010	0.013			0.009
Isocitrate dehydrogenase (NAD)	0.00	<0.001	<0.001			
Isocitrate dehydrogenase (NADP)	0.034	0.038	0.149			0.078
α-Ketoglutarate dehydrogenase	0.011	0.008	0.014	0	0.0008	0.013
Succinate dehydrogenase	0.00057	0.086	0.045	0.0075	0.07	+
Fumarate hydratase	0.48	0.003	0.057			0.006
Malate dehydrogenase (NAD)	0.54	1.69	4.80	0.028	1.6	1.095
Malate dehydrogenase (NADP)	0.033					
Malate synthase	0.00	0.150	0.112			
Isocitrate lyase	0.00	0.008	0.008			
NADH oxidase	0.0032			0	0.012	0.002

[a] Expressed as micromoles of substrate oxidized per minute per milligram of protein.
[b] *Hydrogenomonas* sp.–facultative autotroph, grown autotrophically, from Trüper (33).
[c] *Hydrogenomonas* sp.–facultative autotroph, grown heterotrophically, from Trüper (33).
[d] *T. thioparus*–obligate autotroph, from Smith et al. (29).
[e] *T. intermedius*–facultative autotroph, grown autotrophically, from Smith et al. (29).
[f] *N. agilis*–obligate (?) autotroph, grown autotrophically, from Smith and Hoare (28).

succinate dehydrogenase in these cells would, a priori, limit the heterotrophic growth rate of such organisms. This suggests that even if the cells could be grown heterotrophically, they would grow faster autotrophically than heterotrophically. Thus, a recent report (27) and studies in our own laboratory, substantiating that *N. agilis* grows much faster autotrophically than heterotrophically, are not suprising. It may be possible to grow *N. oceanus* heterotrophically, but this has not been experimently verified. The low specific activity of succinate dehydrogenase, regardless of other factors, should limit the growth rate of both of these nitrifying bacteria.

These observations suggest that nitrifying bacteria have lost their ability to utilize organic molecules as primary energy sources during the course of evolution but still maintain their metabolic potential for a heterotrophic existence. Such is not the case with *T. thioparus* (Table 2), which lacks NADH oxidase. The latter organism may represent a more advanced stage of biochemical evolution, in respect to its intermediary metabolism, than is found in *N. oceanus* and *N. agilis*.

Anaplerotic enzyme system. Even if all of the tricarboxylic enzymes were present and functional, they would not meet the biosynthetic needs of this organism. A system is required to replenish the intermediates of the tricarboxylic acid cycle used in biosynthesis. Such systems have been termed "anaplerotic" by Kornberg (18). The types of anaplerotic sequences known include carbon dioxide fixation and the glyoxylate cycle. The latter requires the activity of certain tricarboxylic acid cycle enzymes in conjunction with two additional enzymes, malate synthase and isocitrate lyase. Neither of these anaplerotic enzymes was found in cell-free extracts of *N. oceanus* (Table 2).

A more widespread system by which C_4-compounds are generated is the combination of carbon dioxide with a C_3-compound. In the cell-free extracts of *N. oceanus*, there is an active system which fixes carbon dioxide in the presence of phosphoenolpyruvate (Table 3). This system was markedly stimulated by acetyl CoA and inhibited by ATP and adenosine diphosphate (ADP). The enzyme responsible is believed to be phosphoenolpyruvate (PEP) carboxylase, known to be inhibited by ADP (31), rather than phosphopyruvate carboxykinase which would be stimulated by ADP. Stimulation of PEP carboxylase by acetyl CoA has been reported by Cánovas and Kornberg (3) in *Escherichia coli*. The products of carbon dioxide fixation in the presence of PEP and acetyl CoA were examined by paper chromatography. Two products were evident and were tentatively identified by their chromatographic mobilities as oxaloacetate and citrate. Small amounts of carbon dioxide were also fixed in the presence of pyruvate (Table 4).

Formation of acetyl CoA from pyruvate. For an organism to use the products of the reductive

TABLE 3. *Phosphoenolpyruvate-CO_2-fixation by cell-free extracts of N. oceanus*

Additions[a]	CO_2 fixed[b]
None	0
Acetyl CoA	10
PEP	620
PEP + acetyl CoA	1,520
PEP + ATP	78
PEP + ADP	184

[a] Reaction mixture contained: 1.5 mg of protein, 100 μmoles of Tris (*p*H 8.0), 10 μmoles of $MgCl_2$, 5 μmoles of glutathione, 10 μc of $NaH(^{14}CO_3)$. The following compounds were added where indicated: 5 μmoles of PEP, ADP, or ATP, and 0.1 μmole of acetyl CoA; total volume, 1 ml.

[b] Results expressed as micromoles of CO_2 fixed per minute per milligram of protein.

TABLE 4. *Stimulation of phosphoenolpyruvate-CO_2-fixation by a mixture of pyruvate, CoA, thiamine pyrophosphate, and NAD*

Additions[a]	CO_2 fixed[b]
None	2
PEP	620
PEP + TPP + NAD + CoA + pyruvate	2,860
PEP + acetyl CoA	1,520
PEP + TPP + NAD + CoA	320
TPP + NAD + CoA + pyruvate	78

[a] The reaction mixture was identical to that described in Table 3, and the amounts of added PEP and acetyl CoA were the same. The following were added where indicated: 5 μmoles of pyruvate and NAD, 0.5 μmole of TPP, and 0.1 μmole of CoA.

[b] Results expressed as micromoles of CO_2 fixed per minute per milligram of protein.

pentose phosphate cycle and the Embden-Meyerhof pathway in the tricarboxylic acid cycle, it is necessary that pyruvate be converted to acetyl CoA. The presence of such a system in cell extracts of *N. oceanus* was demonstrated indirectly. The stimulation of the PEP carboxylase system by acetyl CoA provided a sensitive test for acetyl CoA. It was found that a mixture of pyruvate, CoA, NAD, and thiamine pyrophosphate (TPP) produced the same stimulation as acetyl CoA (Table 4). In the presence of appropriate cofactors, this organism is apparently capable of forming acetyl CoA from pyruvate.

An extensive study of *N. oceanus* showed that its growth was not stimulated by the addition of organic compounds to the medium (36). We determined that whole cells did not consume oxygen when incubated with acetate, citrate, glucose, glutamate, isocitrate, lactate, malate, or succinate. Only ammonia promoted an increase in oxygen consumption (10.5 μmoles per min per g of cells) above that of the endogenous rate. The complete system consisted of 100 ml of seawater (*p*H 7.8) containing 100 mg (wet weight) of washed cells and the substrate at a concentration 10^{-4} M. Measurements were made at room temperature.

Phosphofructokinase appeared to be the only enzyme which may be absent from either the Embden-Meyerhof pathway or the tricarboxylic acid cycle. Even if this enzyme is absent, the presence of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, demonstrated in this investigation (Table 1), and the other enzymes of the pentose phosphate pathway, demonstrated by Campbell et al. (2), would allow *N. oceanus* to direct glucose and similar molecules into the tricarboxylic acid cycle. The absence of phosphofructokinase should not prevent these molecules from serving as both carbon and energy sources for *N. oceanus*.

Because of the findings of Smith et al. (29), we thought that *N. oceanus* might lack NADH oxidase, but this enzyme was demonstrated to be present (Table 2). The logic of Smith et al. (29), concerning the improbability of an obligate chemoautotrophic organism having this enzyme, was convincing; we therefore tested two other pure strains of *N. oceanus* and four pure strains of *Nitrosomonas* spp. (both terrestrial and marine) for NADH oxidase. This enzyme was found in all of these ammonia-oxidizing bacteria.

Kelly (14) found that *T. neapolitanus* could use acetate as a major source of carbon for synthesis of lipids, glutamate, arginine, proline, and leucine. For acetate incorporation, this organism required the energy from thiosulfate oxidation and the presence of carbon dioxide. Kelly's results indicated that some of the enzymes intermediate between α-ketoglutarate and oxaloacetate were missing, and he suggested that the Krebs cycle in *T. neapolitanus* was used primarily for glutamate synthesis rather than for fulfilling energy and carbon needs. We have demonstrated all the enzymes of the tricarboxylic acid cycle; thus, it is obvious that *N. oceanus* and *T. neapolitanus* do not share a common basis for obligate autotrophy.

Likewise, *N. oceanus* does not share a common biochemical basis for obligate autotrophy with the blue-green algae and the *Thiobacilli* spp. studied by Smith et al. (29). Although no doubt exists concerning *N. oceanus* having NADH oxidase, its presence would seem to be incompatible with an obligate chemoautotrophic existence. As pointed out by Smith et al. (29), this

organism, along with many other obligate chemoautotrophic bacteria, cannot reduce NAD by electrons derived from the oxidation of ammonia or other inorganic substrates. Since reduced pyridine nucleotides are needed for CO_2 fixation, the cells most likely generate NADH by the ATP-mediated reversal of electron transport. In view of this, it seems, a priori, that the lack of NADH oxidase would conserve the reduced pyridine nucleotide formed by the expenditure of energy for CO_2 fixation. As NADH oxidase is present in *N. oceanus*, the cells must have some other control mechanism to prevent NADH oxidation.

Uptake of ^{14}C-labeled organic substrates. Initially, in our permeability studies, we conducted short-term experiments with resting cells. It soon became apparent that the bulk of the radioactivity measured resulted from the adsorption of the ^{14}C-labeled organic molecule onto the membrane filter. Nevertheless, a significant amount of radioactivity was found in the trichloroacetic acid-soluble and -insoluble fractions of cells after 2 hr of incubation with ^{14}C-glutamate (Table 5). As the radioactivity remained with the trichloroacetic acid-insoluble fraction after 10 washings with unlabeled glutamate, it was apparent that the measured radioactivity in this fraction resulted from incorporation rather than adsorption.

Chromatographic examination of the trichloroacetic acid-soluble fraction furthermore revealed the presence of two radioactive compounds, one of which was glutamate, thus indicating a biological conversion of glutamate to at least one other compound. This other compound had the same chromatographic mobility as proline.

Owing to the limited amount of incorporation of ^{14}C-labeled compounds in short-term experiments, cells were grown in 1-liter fermentors in the presence of small amounts of labeled organic compounds with known specific activities. Because *N. oceanus* has a generation time of 24 hr or more, the cells were grown 10 to 12 days in the presence of the labeled organic compounds.

The percentage of incorporation of ^{14}C-labeled compounds into the trichloroacetic acid-insoluble fraction of *N. oceanus* during growth experiments is given in Table 6. A more detailed study was made of the trichloroacetic acid-soluble and -insoluble fractions from cells grown with labeled glutamate. The trichloroacetic acid-insoluble fraction was hydrolyzed, the glutamate was separated from the hydrolysate, and its specific radioactivity was determined. In addition, the final specific activity of the glutamate in the medium was measured to establish whether there had been extensive excretion or exchange of glutamate during the course of the experiment. The specific activity of the medium remained the same; activities found in the trichloroacetic acid-soluble and -insoluble fractions are given in Table 7.

TABLE 5. *Distribution of radioactivity in cells of N. oceanus after 2 hr of incubation with ^{14}C-glutamate*[a]

Fraction	Radioactivity
	counts/min
Trichloroacetic acid-soluble	2,160
Trichloroacetic acid-insoluble	216

[a] Cells (1.25 mg, wet weight) in 0.25 ml of seawater were incubated with 1.0 μmole of ammonium sulphate and 0.2 μmoles (1.06×10^6 counts/min) of ^{14}C-glutamate.

TABLE 6. *Incorporation of exogenous ^{14}C-labeled substances into the trichloroacetic acid-insoluble fraction of N. oceanus during growth*[a]

Substrate	Radioactivity in trichloroacetic acid-insoluble extract[b]
^{14}C-glucose	0.003
^{14}C-glutamate	0.16
[*methyl*]-^{14}C-methionine	0.28
Pyruvate-3-^{14}C	3.7

[a] The concentration of radioactive glucose, glutamate, and pyruvate was approximately 10 μc (10 μmoles) per liter; the concentration of methionine was 2 μc (0.1 μmole) per liter. The cultures yielded about 10 mg of protein per liter.

[b] Expressed as per cent incorporation of added material.

These data indicate that only a small percentage of either the free intracellular glutamate or the incorporated glutamate came from an exogenous pool. The fact that the specific radioactivity of the incorporated glutamate is higher than that of the free intracellular glutamate is probably not significant and is in the realm of experimental error. Since the cell is synthesizing over 99% of its glutamate by CO_2 fixation, it seems unlikely that glutamate supplied at the concentration used in these experiments can contribute significantly to the carbon needs of the cell.

A similar situation exists with methionine. Although the trichloroacetic acid-soluble fraction was used for experiments not reported here, we did examine the insoluble fraction; these results are given in Table 8. The label was found both in phosphatidylcholine and in the protein fraction. As with the case of glutamate, only an insignificant amount of the methionine in these fractions came from the exogenously supplied methionine.

Under these experimental conditions, glucose, glutamate, and methionine cannot be used to

TABLE 7. *Specific radioactivities of free and incorporated glutamate in cells of N. oceanus grown in the presence of ^{14}C-glutamate*

Location of glutamate	Specific radioactivity (μc/μmole)	Dilution of label
Added to medium	0.68	
In medium after harvesting	0.61	0
Free intracellular	0.50×10^{-3}	1,400
Incorporated into cellular material	1.7×10^{-3}	380

TABLE 8. *Specific radioactivities of a phosphatidylcholine and incorporated methionine in cells of N. oceanus grown in the presence of [methyl]-^{14}C-methionine*

Material	Specific radioactivity[a]	Dilution of label
Methionine added to the medium	13.2	
Phosphatidyl choline from the cell lipid	2.5×10^{-3}	5,300
Incorporated methionine	6.7×10^{-3}	1,960

[a] Expressed as microcuries per micromole of carbon in the methyl group.

fulfill the major carbon needs of *N. oceanus*. The cell synthesized these compounds by CO_2 fixation far more rapidly than it took them up from the external medium. *N. oceanus* incorporated significantly more pyruvate than the other three organic compounds tested. But pyruvate, when supplied in concentrations of 10^{-2} to 10^{-6} M (Watson, *unpublished data*), did not stimulate growth, suggesting that it, too, could not act as a major carbon donor for this organism.

Although it would have been desirable to perform parallel incorporation experiments with higher substrate concentrations, we wanted to maintain high specific activities and thus did not dilute the labeled compounds with unlabeled carriers. The concentrations of the organic compounds used were as high or higher than found in seawater, which is the natural habitat of this organism. One of our primary concerns was to determine whether *N. oceanus* growing in the ocean could supplement its carbon needs by growing in the substrate concentrations existing in the ocean. These experiments indicate that such a possibility is unlikely.

These experiments demonstrate that *N. oceanus*, like all other chemoautotrophic bacteria thus far tested, is not completely impermeable to organic molecules, but the rate of entry of the compounds tested is too slow to contribute significantly to the economy of the cells. These experiments do not permit us to generalize that all chemoautotrophic bacteria would be so impermeable to all organic compounds. Yet, most of the obligate autotrophic bacteria examined appear to have permeability barriers restricting the flow of organic molecules into the cells (5, 6, 29).

It seems unlikely that the basis of obligate autotrophy in *N. oceanus* can be explained solely by a metabolic defect. Instead, a permeability barrier at the level of the plasma membrane restricts, although not entirely, the entrance of the four organic compounds tested into the cytoplasm of the cell. The rate of entry of the four compounds tested would not be sufficient to meet or contribute significantly to either the carbon or energy requirement of the cells.

That all chemoautotrophic bacteria lack such a rigid permeability barrier is evident, since acetate supplied 25% of the cellular carbon in *T. neapolitanus* (14) and 33 to 39% in *N. agilis* (28). Thus, the permeability of autotrophs to organic compounds varies greatly. Of all the autotrophs thus far examined, *N. oceanus* has the most pronounced permeability barrier to organic compounds.

Even if *N. oceanus* were less efficient at excluding organic molecules, it seems doubtful, because of the low specific activity of succinate dehydrogenase, that this organism could live heterotrophically. These results, combined with those of others, indicate multiple bases for obligate autotrophism. Specifically, an organism may be obliged to live autotrophically because (i) it lacks one or more critical enzymes needed to grow heterotrophically, (ii) the specific activity of one or more of its enzymes may be too low to permit heterotrophic growth, (iii) organic molecules in the medium may not enter the cells rapidly enough to supply its energy and carbon needs, or because of a combination of two or more of these factors.

It is now clear that intermediate between the obligate autotrophs, which lack a key enzyme, and the facultative autotrophs, which have all the enzymes in sufficient quantity to permit them usually to grow faster heterotrophically than autotrophically, there are other organisms, like *N. agilis* and *N. oceanus*, which do not fit clearly into either of these categories. These bacteria, because of permeability barriers to organic substrates or because of an insufficient quantity of enzymes, grow much better autotrophically than heterotrophically. We propose that such organisms should be referred to as pseudoautotrophs.

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