NITROGEN FIXATION BY AZOTOBACTER IN A NATURAL AQUATIC ENVIRONMENT

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ABSTRACT

NITROGEN FIXATION BY *AZOTOBACTER*
IN A NATURAL AQUATIC ENVIRONMENT

By

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Azotobacter is a nitrogen fixing bacterium. Laboratory studies imply that *Azotobacter* plays a significant role in the nitrogen cycle and consequently it appeared important to study the relative importance of this genus in nitrogen fixation in the natural environment.

Wintergreen Lake at the W. K. Kellogg Biological Research Station was the lake site used for *in situ* experiments. Nitrogen fixation was assayed using the acetylene reduction technique. Viable counts were made. Radioisotopes were used to study respiration and protein synthesis. The glucose level in the lake was estimated using the Hobbie-Wright kinetic analysis of the uptake of radioactive glucose by the natural lake population. Most probable number studies were made to determine the numbers of *Azotobacter* in the lake.

Azotobacter vinelandii appeared not to contribute significantly to nitrogen fixation in Wintergreen Lake water. Studies of viability, respiration, and protein synthesis in lake water indicated that cells added to lake water retained essentially a maintenance level metabolism. Suitable carbon sources appeared to be limiting in the lake, with the glucose concentration only 13.5 µg/liter. Most probable number studies

showed that aerobic, heterotrophic nitrogen fixers were present in very low numbers in the lake, about one bacterium in two milliliters of lake water.

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INTRODUCTION

Nitrogen is an essential element. Its transformations in the biosphere are almost completely regulated by aquatic and terrestrial microorganisms. The availability of nitrogen compounds can govern the degree of photoautotrophic development in numerous waters (1). The ability of an organism to fix atmospheric nitrogen, i.e., to convert N_2 to $2NH_3$, may serve as a selective advantage in nitrogen-poor environments.

A nitrogen fixing organism, *Azotobacter*, was isolated by Beijerinck and van Delden in 1902 (3). Later workers have shown that pure cultures of *Azctobacter* actively fix nitrogen in amounts as large as 20 mg per gram sugar (15,46), which is more than double that fixed by most clostridia (1). Although laboratory studies imply that *Azotobacter* plays a significant role in the nitrogen cycle in the environment, definitive *in situ* experiments have not been performed.

Azotobacters are free-living, aerobic, heterotrophic microorganisms widely distributed in soil and water (27,34). They are characterized morphologically by the generally large size of the individual cells which vary considerably in shape and size (34,48). Some azotobacter species have the ability to form cysts, modified vegetative cells with thick coats, under conditions not favorable for vegetative cells (29, 30,34). Considerable information is available concerning the morphology, growth requirements, carbon metabolism, and utilization of nitrogen

sources by *Azotobacter* (7,8,9,10,11,13,15,16,17,18,19,21,22,26,34,35, 39,41,45,48,51).

The specific influence of a combined nitrogen source on nitrogen fixation varies with the quantity and quality of combined nitrogen present. *Azotobacter* will both fix nitrogen and utilize ammonia nitrogen when the latter is present in low concentrations, i.e., 150 mg ammonium per liter (8,39,42). *Azotobacter* will also fix nitrogen when low levels of nitrate are present, provided molybdenum and vanadium are present in quantities sufficient for nitrogenase synthesis; in the absence of these metals, only nitrate is used (5,21). Higher concentrations, i.e., 410 mg/liter, of ammonium inhibit nitrogen fixation by *Azotobacter* (8,11,26,39). The order of preference of utilization of nitrogen sources is: (1) ammonia, (2) urea, (3) nitrite, (4) nitrate, and (5) atmospheric nitrogen (10,39). Therefore, one would expect *Azotobacter* to be fixing nitrogen in significant amounts only when a source of combined nitrogen is unavailable. Organic nitrogen compounds, except urea, are utilized less readily than ammonium, nitrate, and free nitrogen (19).

Azotase is the enzyme complex in *Azotobacter* which catalyzes the fixation of molecular nitrogen, the amount of nitrogen fixed being directly proportional to the metabolic activity of the cell (7). *Azotobacter* requires phosphorous, sulfur, potassium, calcium, magnesium, iron, and molybdenum, in addition to some form of nitrogen and an energy source, for growth. If one of these substances is present at suboptimal concentrations or lacking, the organism will have a reduced growth rate or exhibit no growth, consequently showing a decreased rate of nitrogen fixation or complete absence of nitrogen fixation (4,7,18, 22,23,26,38). Cobalt has been shown to stimulate the amount of nitrogen

fixed (24). *Azotobacter* can utilize a wide variety of carbon sources (13,15,17,26,41).

Nutritional requirements alone do not determine the ability of *Azotobacter* to grow and fix nitrogen. Other factors such as temperature, oxygen concentration, pH, and interaction with other organisms combine with nutrient levels to form a dynamic system in which conditions can be favorable or unfavorable for *Azotobacter* growth and nitrogen fixation. Azotobacters are mesophilic with minimum, optimum, and maximum temperatures for growth of approximately 10, 30, and 40-45 C (26). The temperature optimum may vary depending on the nitrogen source (7). The nitrogen-fixing efficiency of *Azotobacter* increases with a decrease in pO_2 (36,42). The optimal pH for *Azotobacter* growth is around pH 7.2-7.6 (51.). The minimal pH at which growth can still be maintained varies from pH 5.5-6.5, depending on the strain (39). Nitrogenase, the nitrogen fixing component of the azotase complex, functions best over a fairly narrow pH range near pH 7.0, and fixation falls off markedly above and below about pH 6.5-7.0 (9). It was reported that nitrogen fixation by *Azotobacter* was stimulated by the presence of associated organisms (26), but Lind and Wilson (31) concluded that media or the techniques or both were unsatisfactory for the best development of the organism. The role of associated microorganisms may be only to alter the medium so that it becomes more suitable for the growth of the *Azotobacter*. Since heterotrophic nitrogen fixation depends on the availability of exogenous organic carbon, the presence of other organisms may also have a negative effect on growth and nitrogen fixation by *Azotobacter*. In the natural environment, *Azotobacter* may not be able to successfully compete for available carbon sources with other nitrogen-fixing organisms, and also with non-nitrogen-fixing forms.

In the present study, the ability of *Azotobacter vinelandii* (ATCC no. 12837) to fix nitrogen in Wintergreen Lake water was investigated in an attempt to determine the importance of *Azotobacter vinelandii* in the nitrogen cycle of the lake. The studies established that *Azotobacter vinelandii* does not fix nitrogen in Wintergreen Lake water and therefore probably does not contribute significantly to the nitrogen cycle.

MATERIALS AND METHODS

Lake Site

Wintergreen Lake is a eutrophic lake at the W. K. Kellogg Biological Research Station, Hickory Corners, Michigan.

Assay for Nitrogen Fixation

Nitrogen fixation was assayed indirectly by a modified acetylene reduction technique of Stewart, Fitzgerald, and Burris (43).

(1) Gases. Acetylene (purified grade), a gas mixture of O_2 (22%), CO_2 (0.04%), and argon (78%, high purity), and certified ethylene standards of 47 ppm and 830 ppm ethylene in nitrogen were obtained from the Matheson Co.

(2) General procedure. Experiments were carried out in 6.0 ml glass serum bottles fitted with rubber serum stoppers. Two milliliters of the sample were added to each bottle. Air was removed by flushing the liquid with the premixed Ar, O_2, CO_2 gas phase for about one minute, and each bottle stoppered. After injection of 0.5 ml of acetylene by means of a glass syringe, the samples were incubated for one hour at 25 C in the laboratory or in the lake. Reactions were terminated by the injection of 0.3 ml of 2.0% $HgCl_2$ (w/v). Samples were assayed in duplicate, along with one zero-time control. The atmosphere in the headspace was analyzed for ethylene produced. The amount of ethylene produced resulted from a one hour incubation time;

however, it was recorded as the amount produced at the initial time of sampling.

(3) Analysis. Ethylene concentration was determined with a Varian-Aerograph Model 600D gas chromatograph equipped with a hydrogen-flame ionization detector. The column used for all analyses consisted of a 1 m x 3 mm (OD) stainless steel column packed with Porapak N, 100-120 mesh (Anspec, Ann Arbor, Michigan). The oven was not heated though the operating temperature stabilized at 45 C. Carrier gas flow (high purity N_2) was approximately 25 ml/min. Compressed air, at a flow rate of 300 ml/min, and H_2, at a flow rate of 25 ml/min, were used to operate the flame detector. Quantities of ethylene produced were determined from a standard curve relating peak height to quantity of ethylene which was prepared using the ethylene standards.

Bacterial Cultures

Azotobacter vinelandii, ATCC no. 12837, *A. chroococcum*, ATCC no. 7499, *A. agilis*, ATCC no. 12838, and two strains of *A. chroococcum* isolated from Wintergreen Lake were used in this study.

Correlation of Ultrastructure with Nitrogen Fixation

Azotobacter vinelandii, ATCC no. 12837, or *A. chroococcum*, ATCC no. 7499, was grown in each of two 250 ml Erlenmeyer flasks. One flask contained 50 ml of Burk's nitrogen free buffer plus 1% glucose (47), and the other, 50 ml of Burk's buffer plus 1% glucose supplemented with 0.25% NH_4Cl as a source of combined nitrogen. The flasks were incubated at room temperature (approximately 25 C) without shaking. The cells were sampled during exponential growth and tested for acetylene reduction. At the same time, the remaining cells were collected on

22 mμ membrane filters and washed with 3 ml of 3% (v/v) glutaraldehyde fixative in phosphate buffer, pH 7.2. A thin layer of 1% Noble agar was placed on the filters which were then immersed in the glutaraldehyde fixative and kept at 4 C overnight. The filters were rinsed with cold phosphate buffer four times during the next day, sectioned into small pieces, and post-fixed overnight in 1% osmium tetroxide buffered at pH 6.1 with Veronal acetate buffer (28) at room temperature. The fixed preparations were then soaked in 0.5% uranyl acetate solution for approximately 2 hours and dehydrated in a graded series of ethyl alcohol and propylene oxide. Pieces were then embedded in Epon 812 by the method of Luft (32). The blocks were cured for 24 hours at 45 C and then for 24 hours at 60 C. Thin sections of the cured blocks were cut on an LKB III ultramicrotome (LKB Instruments, Copenhagen, Denmark) with a DuPont diamond knife and mounted on 300 mesh athene type copper grids. Sections were doubly stained, first with 2% uranyl acetate for 10 minutes and then in lead citrate for 10 minutes. After being stained, the samples were examined with a Hitachi electron microscope (HU-11) (Hitachi, Ltd., Tokyo, Japan).

Isolation and Classification of *Azotobacter* from Wintergreen Lake

Enrichment media were used to isolate *Azotobacter* from Wintergreen Lake. Samples were obtained aseptically (see Appendix) from Wintergreen Lake in January 1971 from depths of 5 cm, 1 m, 2 m, and 3 m. A sample was collected from the mud-water interface at approximately a 5 m depth with a van Dorn sampler and placed in a sterile container. One milliliter of each sample was pipetted into each of 4 500-ml Erlenmeyer flasks containing 100 ml of Burk's nitrogen-free buffer plus one of the following carbon sources: glucose 0.1%, resorcinol 0.1%, mannitol

0.1%, or ethanol 1.0%. The flasks were incubated for 8 days at room temperature (approximately 25 C). A duplicate set of flasks was incubated at 12 C, the temperature of the surface water of the lake at the time of collection. Uniform turbidity was observed in the flasks incubated at 25 C containing 0.1% glucose or 1.0% ethanol inoculated with the mud-water interface sample; growth did not occur in any of the other enrichment flasks. The cultures were examined microscopically for typical *Azotobacter* cells. One and one-half percent agar plates of Burk's buffer plus an added carbon source were used as the solid medium for streak plates. Solid medium plus 1% glucose was streaked with each of these cultures and incubated at 25 C. Isolated colonies were streaked for purity on solid medium plus 1% glucose. The isolates, one from the ethanol enrichment and one from the glucose enrichment, were streaked along with known ATCC cultures of *Azotobacter vinelandii*, *A. chroococcum*, and *A. agilis* on solid medium that contained mannitol, ethylene glycol, resorcinol, butanol, or starch. A single streak was made of each organism. Organisms growing on butanol were checked microscopically for cyst production. The isolates were assayed for their ability to reduce acetylene during growth.

Growth of the Two Isolates of *Azotobacter chroococcum* from Wintergreen Lake Under Various Partial Pressures of Oxygen

The isolates of *Azotobacter chroococcum* were tested for growth and acetylene reduction under various partial pressures of oxygen. Eight 500 ml side arm flasks containing Burk's nitrogen-free buffer plus 1% glucose were set up with a gas flow apparatus as seen in Figure 18. Certified oxygen standards of 0.1%, 0.95%, 5.0%, and 20.0% oxygen in nitrogen (Matheson Co.) were bubbled through the medium. The flasks were inoculated with stationary phase cells and incubated

on a shaker at 25 C. The flasks were observed periodically for absorbance at 660 nm in a Bausch and Lomb Spectronic 20. Twice during the exponential phase the cultures were sampled and tested for acetylene reduction. The samples were flushed with the same gas that flowed through the culture and quickly capped.

Preparation of Cells Used in Experiments on Nitrogen Fixation in Lake Water, Viability in Lake Water, Repression of Nitrogen Fixation by Ammonium ion, and Nitrogen Fixation During Encystment

Cells of *A. vinelandii* were grown in Burk's nitrogen-free buffer plus 1% glucose at 25 C. Growth was followed by direct microscopic counting in a Petroff-Hausser bacteria counter and by turbidity measurements at a wave length of 660 nm in a Bausch and Lomb Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, New York). Cells in the late exponential phase of growth (about 18 hr) were harvested by centrifugation at 4 C in a RC-2B Sorvall refrigerated centrifuge at 3000 x g for 20 minutes, washed with water, and suspended in the appropriate solutions.

Preparation of Bacteria-Free Lake Water

Lake water was obtained from the surface of Wintergreen Lake in sterile containers. The water was passed through sterile 0.45 μm followed by sterile 0.22 μm membrane filters (Millipore Corp., Bedford, Mass.).

Nitrogen Fixation in Lake Water

Cells suspended in sterile water were dispensed in 2-ml volumes into 12 100-ml glass serum bottles. Six of the bottles contained 48 ml of bacteria-free lake water, and 6 contained 48 ml of Burk's nitrogen-free buffer. The final cell concentration was 2×10^9 cells/ml.

Glucose was added to each of the 6 bottles to final concentrations of 0, 1, 10, 100, 1,000, and 10,000 µg/ml. Nitrogen fixation was assessed at zero time and at 3 hours. Zero and 3 hours are the times at which the samples were taken for the assay; however, the assay takes one hour. The data reflect the cell response over a 1-hour period. The amount of ethylene produced per hour at zero time was subtracted from the amount of ethylene produced at 3 hours to indicate a decrease or increase in the rate of nitrogen fixation. The amount of ethylene produced per hour in Burk's nitrogen-free buffer plus 10,000 µg glucose per milliliter was taken to be the maximum amount of fixation under optimum conditions. The ethylene produced in the other samples is calculated as a percentage of this maximum amount.

Viability in Lake Water

Cells were suspended in bacteria-free lake water or Burk's nitrogen-free buffer to a final concentration of 20 cells per milliliter. At intervals of 0, 4, 8, 12, and 48 hours, samples were withdrawn and 1:4 dilutions made in sterile water blanks. Triplicate 0.2 ml samples were plated on Burk's nitrogen-free agar (1.5%) plus 1% glucose. Colonies were counted after the plates were incubated at 25 C for 24 hours. The generation time, g, was calculated according to the formula: $g = t(\log 2)/\log b - \log a$, where a is the initial number of cells, b is the number of cells after time t, and t is the time interval between measurements.

Radioisotopes

Glucose-U-^{14}C and L-leucine-U-^{14}C were obtained from the New England Nuclear Corporation, Boston, Mass.

Respiration Studies

Azotobacter vinelandii was grown in 100 ml of Burk's nitrogen-free buffer plus 1% glucose plus 10 µc glucose-U-^{14}C (specific activity 207 µc per µmole) for 18 hours. The cells were harvested by centrifugation at 20,000 x g for 20 minutes and resuspended in triplicate 50 ml volumes of bacteria-free lake water or sterile Burk's nitrogen-free buffer in 500-ml Erlenmeyer flasks to a final concentration of about 2 x 10^{10} cells per milliliter. These 6 flasks were incubated at 30 C in a waterbath shaker. One-half milliliter samples were withdrawn from each flask at 30-minute intervals and measured for radioactivity in a liquid scintillation counter (Packard Tri-Carb scintillation spectrometer model 3310) using the following procedures:

(1) Cells. The cell suspension was collected on 0.45 µm membrane filters (Millipore Corp., Bedford, Mass.) and washed with 1.5 ml of nonradioactive buffer or lake water. Filters were dried and placed in 5.0 ml of toluene base scintillation fluid (12).

(2) Supernatant. The filtrates were combined with the washings and assayed in scintillation vials containing 10 ml of Bray's solution (6).

(3) CO_2. $^{14}CO_2$ was trapped in fluted Whatman no. 1 filter paper (3 x 5 cm) soaked with 0.25 ml 10% KOH. The filter paper was placed in a scintillation vial which was suspended in the 500-ml Erlenmeyer flask by means of a wire and was changed every 30 minutes. After being dried the paper was placed in 5.0 ml of toluene base scintillation fluid (12). $^{14}CO_2$ counts per minute are recorded as cumulative values.

Protein Synthesis

The synthesis of protein was estimated indirectly by measuring the incorporation of L-leucine-U-^{14}C (specific activity 306 μc/μmole) into trichloroacetic acid-insoluble precipitates following the basic procedure of Yang and Brubaker (52).

Cells, growing in Burk's nitrogen-free buffer plus 1% glucose, were harvested at 18 hours by centrifugation at 20,000 x g in an RC-2B refrigerated centrifuge. The cells were resuspended in fresh bacteria-free lake water or Burk's nitrogen-free buffer to which L-leucine-U-^{14}C was added at a concentration of 5 μc/ml. One-half milliliter samples were removed at 3-hour intervals from the cultures, mixed with 0.5 ml of cold 10% trichloroacetic acid (TCA), incubated for 30 minutes at 5 C, and then collected on 0.45 μm membrane filters. The filters were washed 3 times with 5.0 ml of 5% cold TCA containing 5 μM/ml nonradioactive leucine to displace any ^{14}C-leucine bound to the precipitate. The filters were dried, put into 5.0 ml toluene base scintillation fluid, and counted.

Amount of Glucose in Lake Water

The Hobbie-Wright technique (49), using high specific activity glucose-U-^{14}C and based on Michaelis-Menten enzyme kinetics, was employed to estimate the concentration of glucose in Wintergreen Lake.

To 25-ml quantities of nonfiltered lake water in 100-ml glass serum bottles were added various amounts of glucose-U-^{14}C to give final concentrations of 5, 15, 30, and 45 μg glucose/liter. The bottles were capped with rubber serum caps and incubated in the lake (see Appendix) for 1 hour. Blanks were prepared by adding 45 μg glucose-U-^{14}C/liter immediately followed by I_2-KI (Lugol's solution containing 1 g

iodine, 2 g KI, and 300 ml distilled water) to inhibit metabolic activity. At the end of the incubation period the samples were fixed with 1 ml of the I_2-KI solution. Cells were collected on 0.22 µm membrane filters, placed in 15 ml Bray's solution (6) and counted in a scintillation counter. Results were analyzed graphically to determine the maximum glucose concentration.

Repression of Nitrogen Fixation by Ammonium Ion

Cells were added to a final concentration of 2×10^9 cells/ml to each of 5 500-ml Erlenmeyer flasks containing 50 ml of Burk's nitrogen-free buffer plus 1% glucose and ammonium in the following concentrations: 0, 26, 52, 129, and 971 mg/liter. Samples were removed from the flasks at intervals of 0, 1/2, 1, 2, 3, and 4 hours and assayed for acetylene reduction.

Cells were also added to a final concentration of 5×10^9 cells/ml to Burk's nitrogen-free buffer plus 1% glucose. Eight and seven tenths milliliters of the cell suspension were then placed in 26 ml serum bottles and assayed for ethylene production with time. Fifty minutes after the acetylene was injected, ammonium was injected into the vials. Ethylene production with time continued to be monitored. Triplicate vials were prepared with ammonium concentrations of 0, 3.37, 33.7, and 337.0 mg/liter. Water was added to the control.

Nitrogen Fixation During Growth

Stationary phase cells of *A. vinelandii* and the 2 isolates of *A. chroococcum* were inoculated into 500-ml Erlenmeyer flasks containing 200 ml of Burk's nitrogen-free buffer plus 1% glucose to concentrations of 4×10^9 cells/ml for *A. vinelandii* and 1×10^9 cells/ml for each of the *A. chroococcum* isolates. Cells were grown on a rotary shaker

(200 rev/min) at 25 C. Growth was followed by direct microscopic counting in a Petroff-Hausser counting chamber and turbidometrically using a Bausch and Lomb Spectronic 20, wave length 660 nm. Samples were taken periodically and assayed for acetylene reduction.

Cells of the *A. chroococcum* strain isolated from the glucose enrichment were also inoculated into each of 3 500-ml Erlenmeyer flasks containing 200 ml of Burk's buffer plus 1% glucose. The final concentration of cells was about 2×10^9/ml. The ethylene produced was assessed every 2 hours. When the rate of ethylene production approached zero, cells in one flask were centrifuged, washed with sterile water, and resuspended in fresh medium.

Nitrogen Fixation During Encystment

Cells grown in Burk's nitrogen-free buffer plus 1% glucose were harvested at 18 hours and suspended into each of 2 500-ml Erlenmeyer flasks containing 50 ml of Burk's buffer plus 1% glucose or Burk's buffer plus 0.2% β-hydroxybutyric acid (BHB). Samples were taken periodically and checked for absorbance at 660 nm and for nitrogen fixation.

Most Probable Numbers (MPN) of *Azotobacter* in Wintergreen Lake

Water samples were collected from the surface of Wintergreen Lake over its deepest area. One-milliliter amounts of lake water were diluted with sterile distilled water and inoculated into 5 tubes of Burk's nitrogen-free buffer plus 1% glucose. After incubation at 25 C for 10 days, the tubes were examined macroscopically for the presence of characteristic *Azotobacter* pellicles and microscopically for *Azotobacter* cells. Most probable number estimates were determined from an MPN index (44).

RESULTS

The effect of incubation time on the production of ethylene was studied in order to determine the proper incubation time to use for the assay of nitrogen fixation. Results are presented in Figure 1. One hour was chosen for the length of incubation for the assay since ethylene production was linear with time up to 75 minutes.

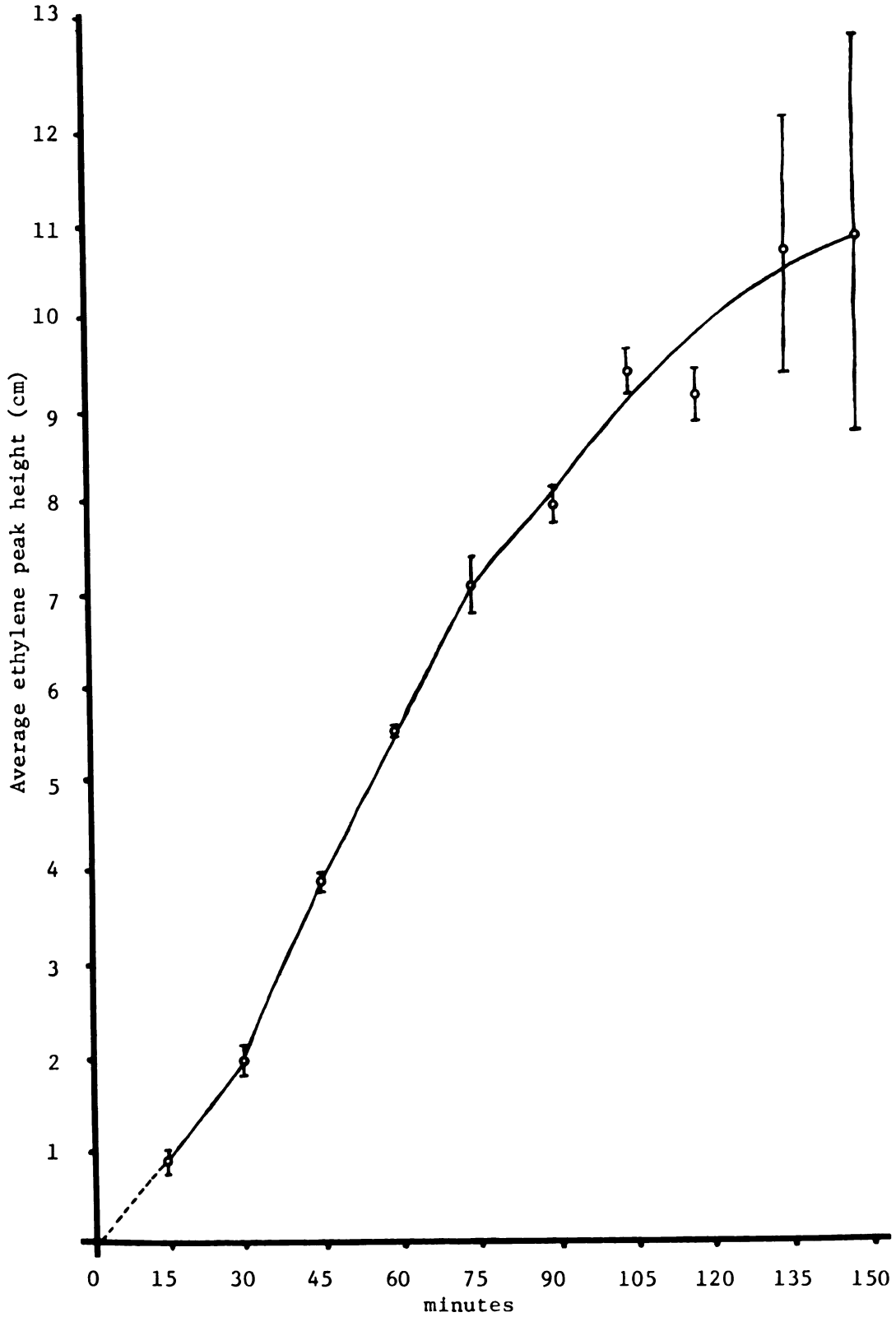
The effect of flushing the incubation chamber, prior to incorporation of acetylene, with an Ar, O_2, CO_2 gas mixture on the amount of ethylene detected is presented in Table 1. The results indicate that approximately 3.2 times more ethylene is detected when the samples are flushed than when they are not flushed.

Table 1. The effect on acetylene reduction of flushing with an Ar, O_2, and CO_2 gas mixture to remove N_2

Sample No.	Hr after inoculation	Avg ethylene peak height (cm)		Ratio of flushed to not flushed
		flushed	not flushed	
1	8	1.45	0.45	3.20
2	10	0.80	0.25	3.20
3	12	4.83	1.35	3.57

Nitrogen fixation in lake water was studied by suspending *Azotobacter vinelandii* in Burk's buffer and comparing the ability of the cells to fix nitrogen with that of cells suspended in lake water. Glucose was added at the indicated concentrations to both the buffer

Figure 1. Ethylene production by *Azotobacter vinelandii* vs time.



and the lake water to determine the effect of added glucose. Ethylene was not produced in either the Burk's buffer or lake water until at least 100 µg glucose/ml was added (Table 2). Even when glucose was added, the amount of ethylene produced in lake water was half the maximum amount produced in Burk's buffer plus 10,000 µg glucose/ml.

Table 2. N_2 fixation by *Azotobacter vinelandii* in lake water: laboratory studies

	Burk's nitrogen-free buffer, pH 6.8		Bacteria-free lake water collected 7/10/71 pH 8.5	
Concn of glucose added in µg/ml	mµmoles of ethylene produced/hr from t_0 to t_{3hrs}*	% max amt of ethylene produced/hr	mµmoles of ethylene produced/hr from t_0 to t_{3hrs}*	% max amt of ethylene produced/hr
0	-0.03		-0.03	
1	-0.03	0.0	-0.03	0.0
10	-0.04	0.0	-0.03	0.0
100	+0.46	112.2	+0.19	46.3
1,000	+0.42	102.4	+0.18	43.9
10,000	+0.41	100.0	+0.20	48.8

*(-) indicates a decrease in ethylene production from the initial amount observed at zero time, (+) indicates an increase in ethylene production from the initial amount observed at zero time.

For samples containing 0, 1, 10 µg glucose/ml the ethylene peak was 2 times that of the dead control.

When this same experiment was repeated 3 months later with the samples incubated in the lake, ethylene production occurred only in Burk's buffer plus 10,000 µg glucose per milliliter, 0.24 mµmoles of ethylene was produced per hour.

Cells growing in Burk's buffer plus 1% glucose were transferred to lake water and buffer in an attempt to determine if the cells were viable and if they were growing. Samples were taken periodically and viable counts made. The generation times of 27.3 hr in Burk's buffer and 29.3 hr in lake water were calculated from the 12- and 48-hr data (Table 3).

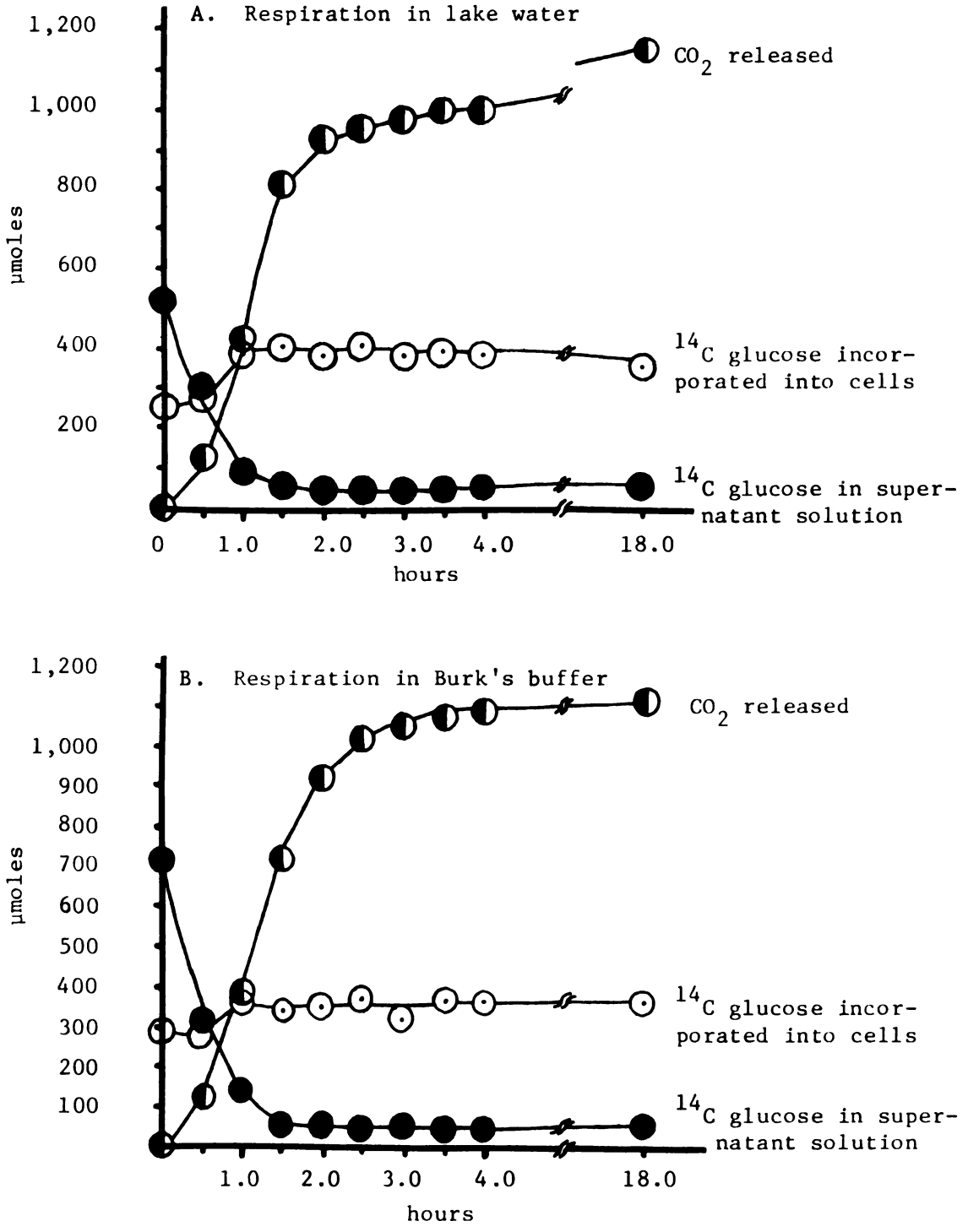
Table 3. Viability of *Azotobacter vinelandii* in lake water

	Hours after inoculation	Average no. of organisms per ml	Generation time in hrs*
Burk's nitrogen-free buffer	0	20	
	4	3,814	
	8	7,460	
	12	5,260	27.3
	48	14,960	
Sterile lake water	0	20	
	4	1,014	
	8	7,014	
	12	10,506	29.3
	48	23,426	

*Calculations were done according to procedures outlined in the Materials and Methods section; generation time was calculated between 12 and 48 hours.

Studies with ^{14}C-glucose grown cells resuspended in lake water or in Burk's buffer were done to determine if the cells respire in lake water. Some ^{14}C-glucose was carried over in the supernatant solution. This was rapidly depleted to a constant level after 2 hours (Figure 2). The respiration rate during the 2- to 18-hr interval after the cells were resuspended in lake water was 1.4×10^{-7} µmoles $^{14}CO_2$ per hour,

Figure 2. Respiration of cells in lake water and in Burk's buffer.



compared to 1.2×10^{-7} μmoles $^{14}CO_2$ per hour in Burk's buffer (Table 4).

The protein synthesizing system was found to be active in cells suspended in lake water (Figure 3). The cells exhibit a greater rate of protein synthesis in lake water than in Burk's buffer. The rate of leucine incorporation was stimulated by the addition of 2 mg/ml glucose to lake water or to Burk's buffer. The rate of protein synthesis in lake water was greater than in Burk's buffer with and without glucose.

The concentration of glucose in the lake was estimated using the kinetic analysis described by Allen (2) (Table 5). A graphical analysis of the data (Figure 4) indicates that the maximum concentration of glucose in Wintergreen Lake is 13.5 μg/liter.

Preliminary studies were done to determine if a correlation could be made between the ultrastructure of *Azotobacter* and nitrogen fixation. The results (Table 6) indicate ethylene was produced in cultures without NH_4Cl and was not produced in cultures with NH_4Cl. Electron micrographs of the organisms can be seen in Figures 5 through 8. Figures 5 and 7 are of thin sections of whole cells of *Azotobacter* grown with N_2, and Figures 6 and 8 are of cells grown with ammonia. Little difference is observed in the internal membrane network in the cells grown with and without NH_4Cl.

Ammonia nitrogen has been reported to repress nitrogen fixation in *Azotobacter* (39). In the present study, as little as 26 mg ammonia nitrogen per liter of Burk's nitrogen-free buffer plus 1% glucose was found to inhibit nitrogen fixation (Figure 9). Higher concentrations of ammonia nitrogen showed the same effect. The effect of ammonia nitrogen on ethylene production with time was studied (Figure 10). Ethylene production was gradually reduced after injection of NH_4Cl.

Table 4. Respiration studies of *Azotobacter vinelandii* in lake water

Hours after inoculation	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	18.0
		μmoles ^{14}C glucose incorporated into cells x 10^8								
Bacteria-free lake water	260	280	390	410	390	410	390	400	390	360
Burk's buffer	290	280	360	340	350	370	320	370	370	360
		μmoles ^{14}C glucose in the supernatant solution x 10^8								
Bacteria-free lake water	520	300	90	60	50	50	50	60	60	60
Burk's buffer	720	310	140	60	60	50	50	50	50	50
				μmoles $^{14}CO_2$ released x 10^8						
Bacteria-free lake water		130	420	810	930	960	980	1000	1010	1160
Burk's buffer		120	380	720	930	1020	1050	1080	1090	1140

The rate of increase in respiration from 2 to 18 hours in lake water is 1.4×10^{-7} μmoles $^{14}CO_2$/hr; and in Burk's buffer, it is 1.3×10^{-7} μmoles $^{14}CO_2$/hr.

Figure 3. Protein synthesis in lake water vs buffer.

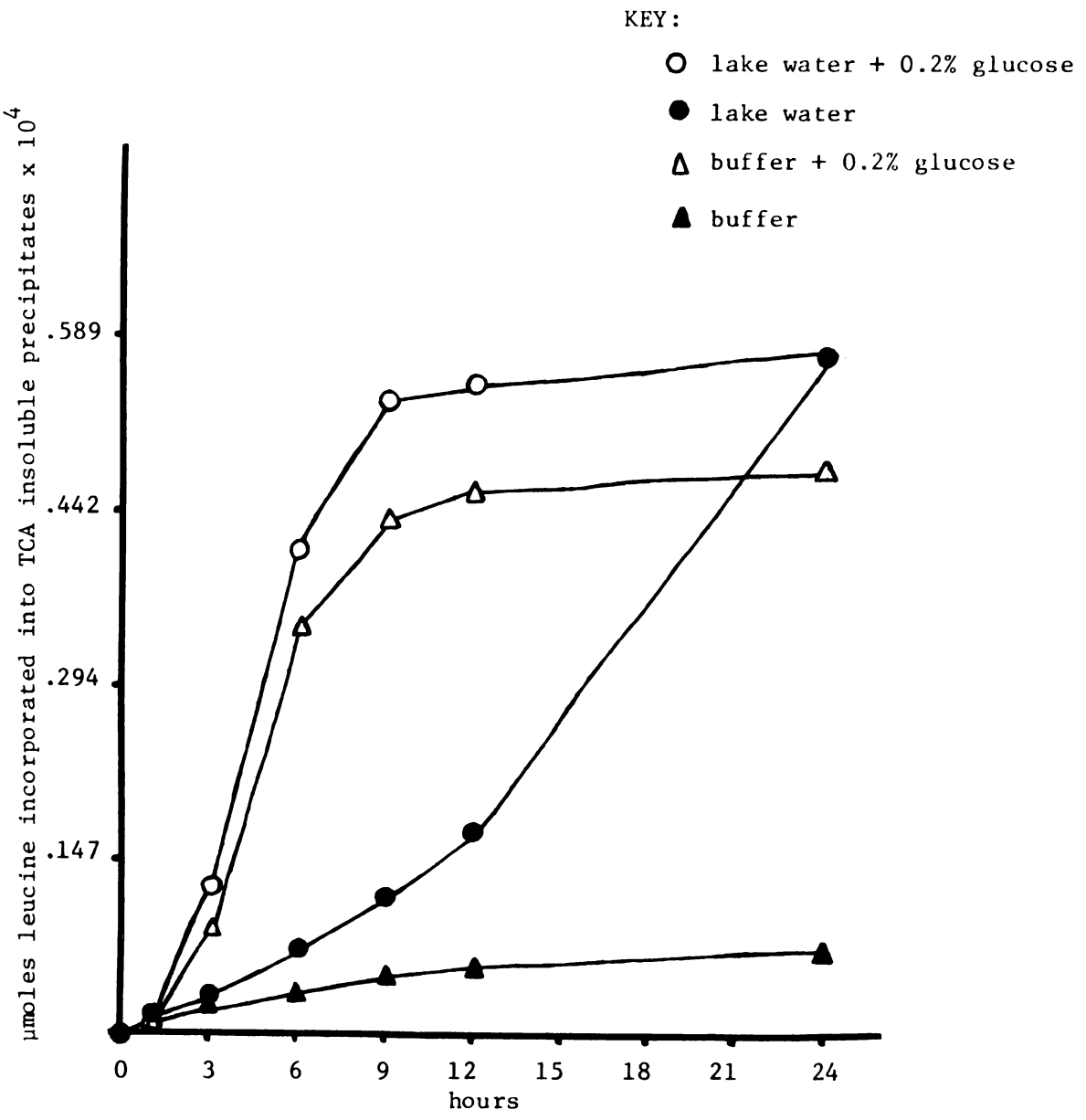


Table 5. Uptake of radioactive glucose by a natural lake population

C, counts/min from 1μc ^{14}C (corrected for machine efficiency	μ, quantity of ^{14}C added to the sample in μg/l	t, incubation time in hours	C, radioactivity of filtered organisms in counts per min*	Cμt/c	A, added substrate in μg/l
177,600 (85% efficiency)	5	1	29,831	29.78	5
	15	1	61,121	43.59	15
	30	1	79,328	67.16	30
	45	1	82,665	96.68	45

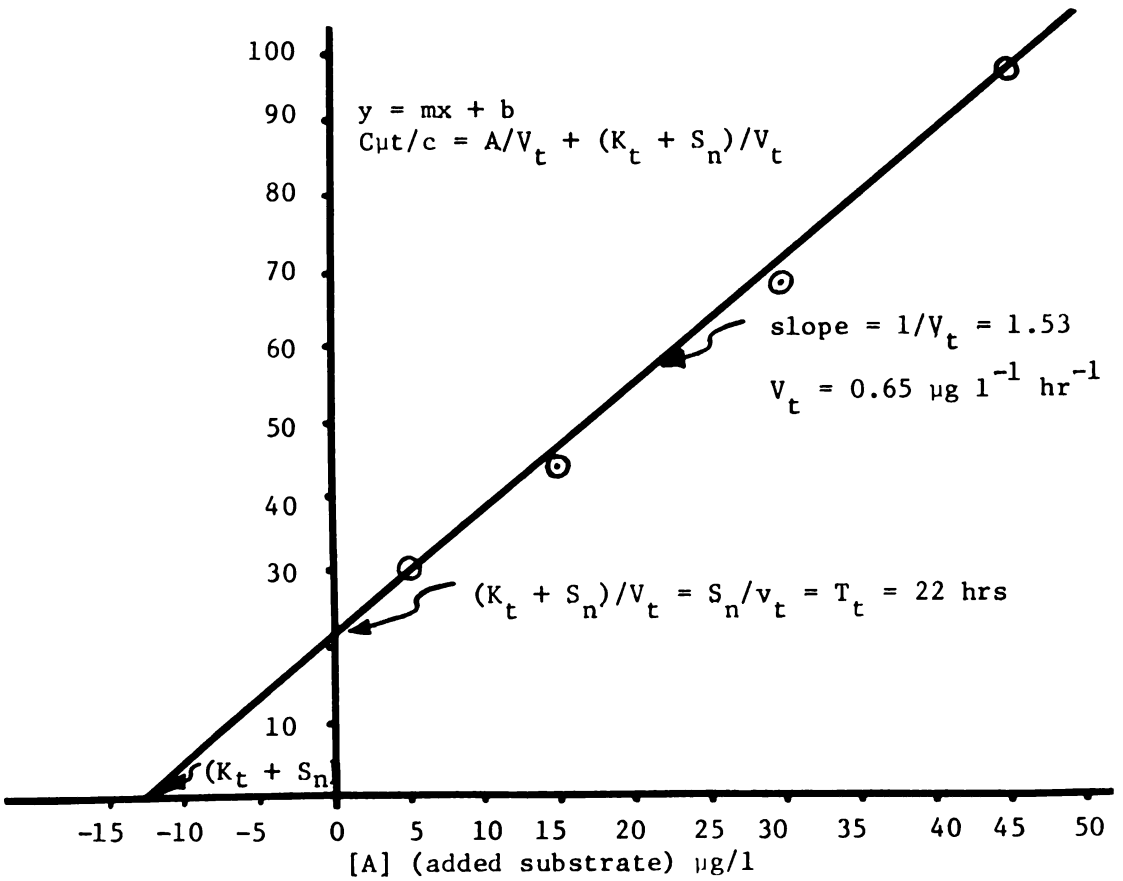
Symbols are those used by Allen (2).

*Counts are corrected for blank and quenching.

Table 6. N_2 fixation by *Azotobacter vinelandii* and *Azotobacter chroococcum* in medium with and without NH_4Cl

Organism	mμmoles ethylene produced/hr	
	Without NH_4Cl	With NH_4Cl
Azotobacter vinelandii	47	0.06
Azotobacter chroococcum	36	0.07

Figure 4. Graphical analysis of bacterial uptake at low substrate concentrations following Michaelis-Menten enzyme kinetics. Plot of Cμt/c against increasing substrate concentrations (s), illustrating derivation of 1) maximum natural substrate concentrations ($K_t + S_n$) as μg/l; 2) maximum velocity of bacterial uptake (V) as μg per liter per hour; and 3) turnover time for substrate regeneration (T_t) as hours.



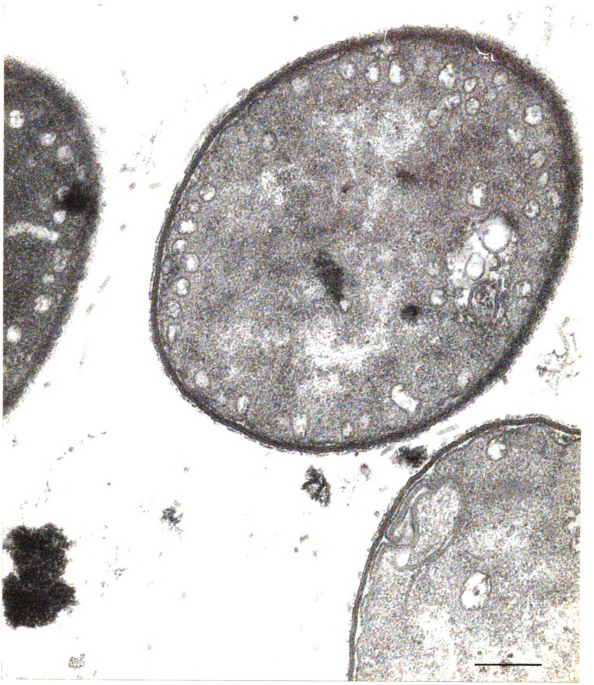


Figure 5. Ultrastructure of *Azotobacter vinelandii* grown with N_2 as the nitrogen source. Total magnification is 87,000X. Bar represents 0.2 μm.

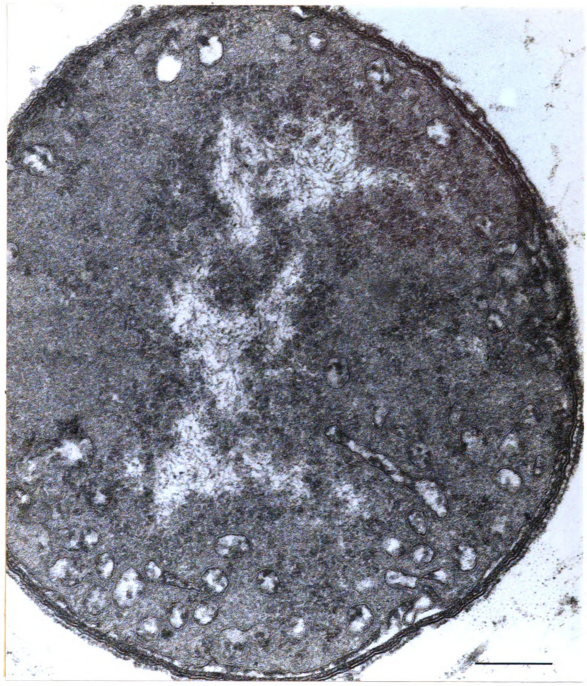


Figure 6. Ultrastructure of *Azotobacter vinelandii* grown with NH_4Cl as the nitrogen source. Total magnification is 100,000X. Bar represents 0.2 μm.

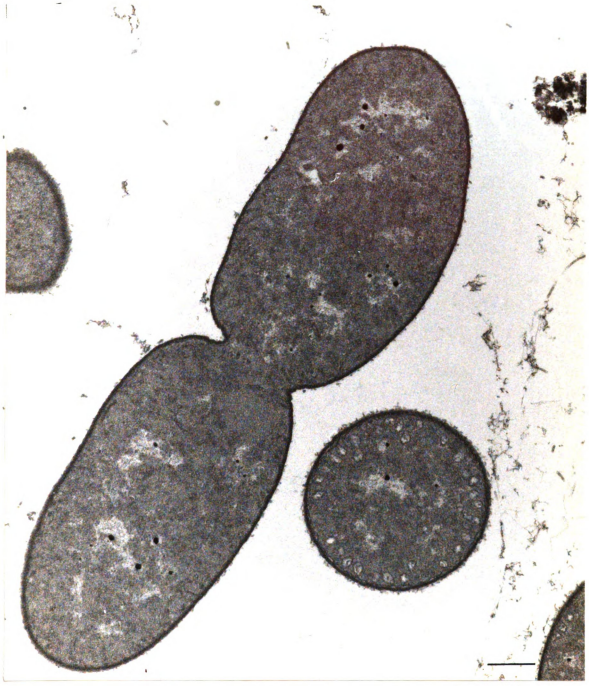


Figure 7. Ultrastructure of *Azotobacter chroococcum* grown with N_2 as the nitrogen source. Total magnification is 34,500X. Bar represents 0.5 μm.

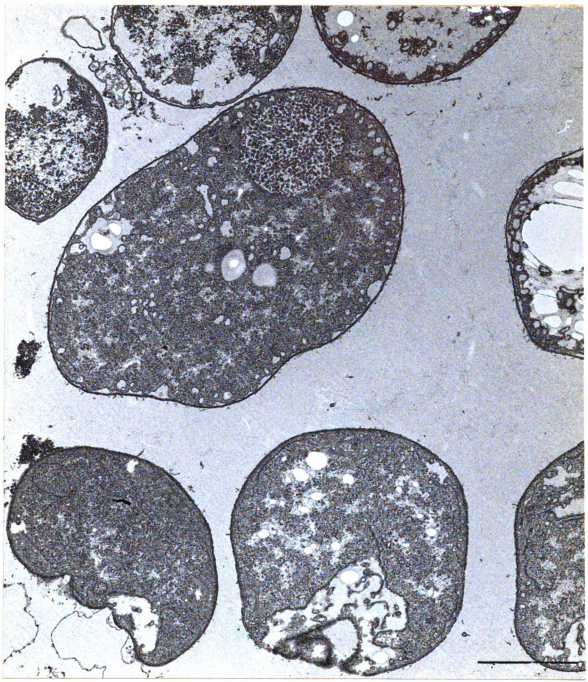


Figure 8. Ultrastructure of *Azotobacter chroococcum* grown with NH_4Cl as the nitrogen source. Total magnification is 26,000X. Bar represents 1 μm.

Figure 9. Repression of ethylene production in *Azotobacter vinelandii* by ammonia nitrogen.

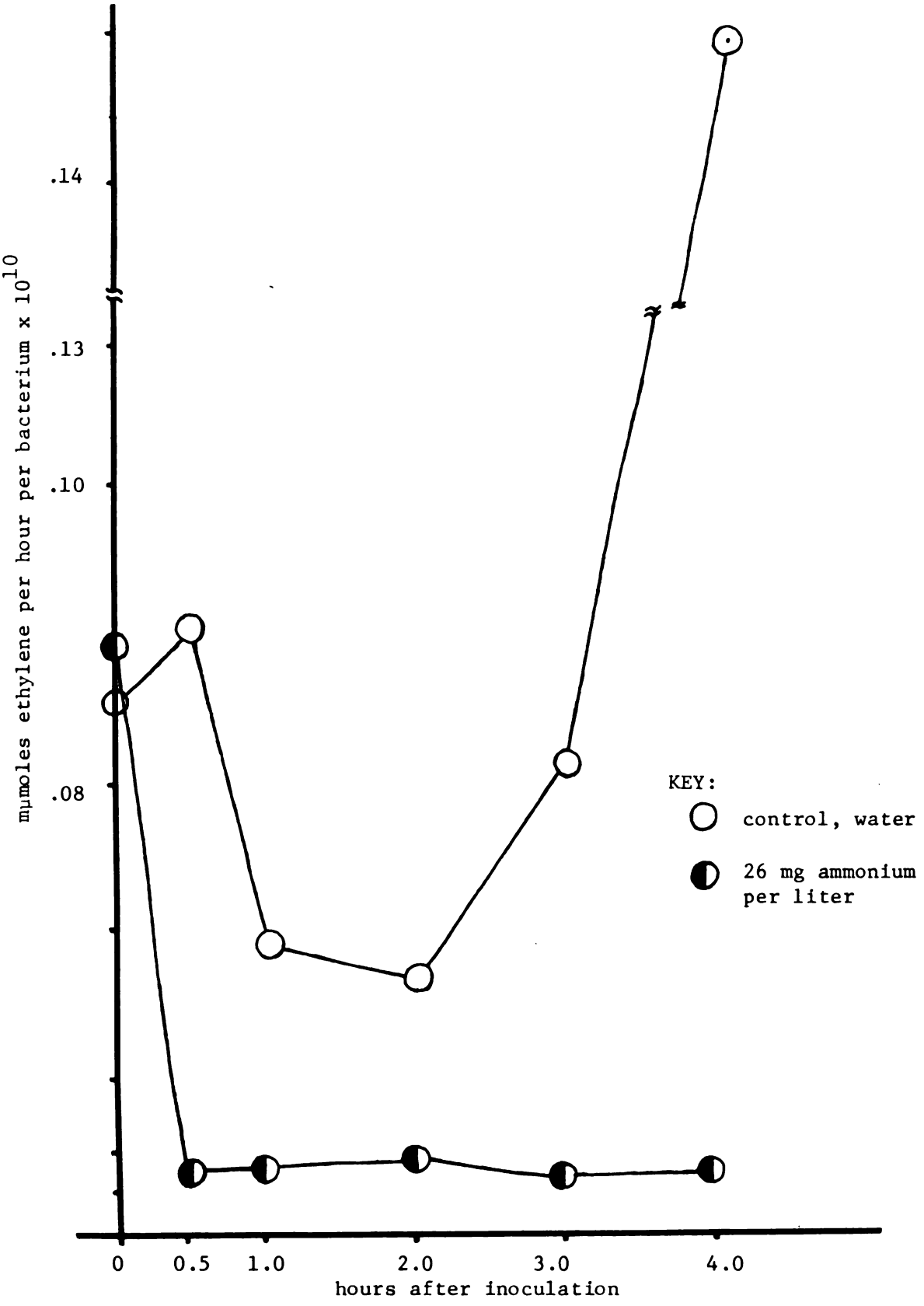
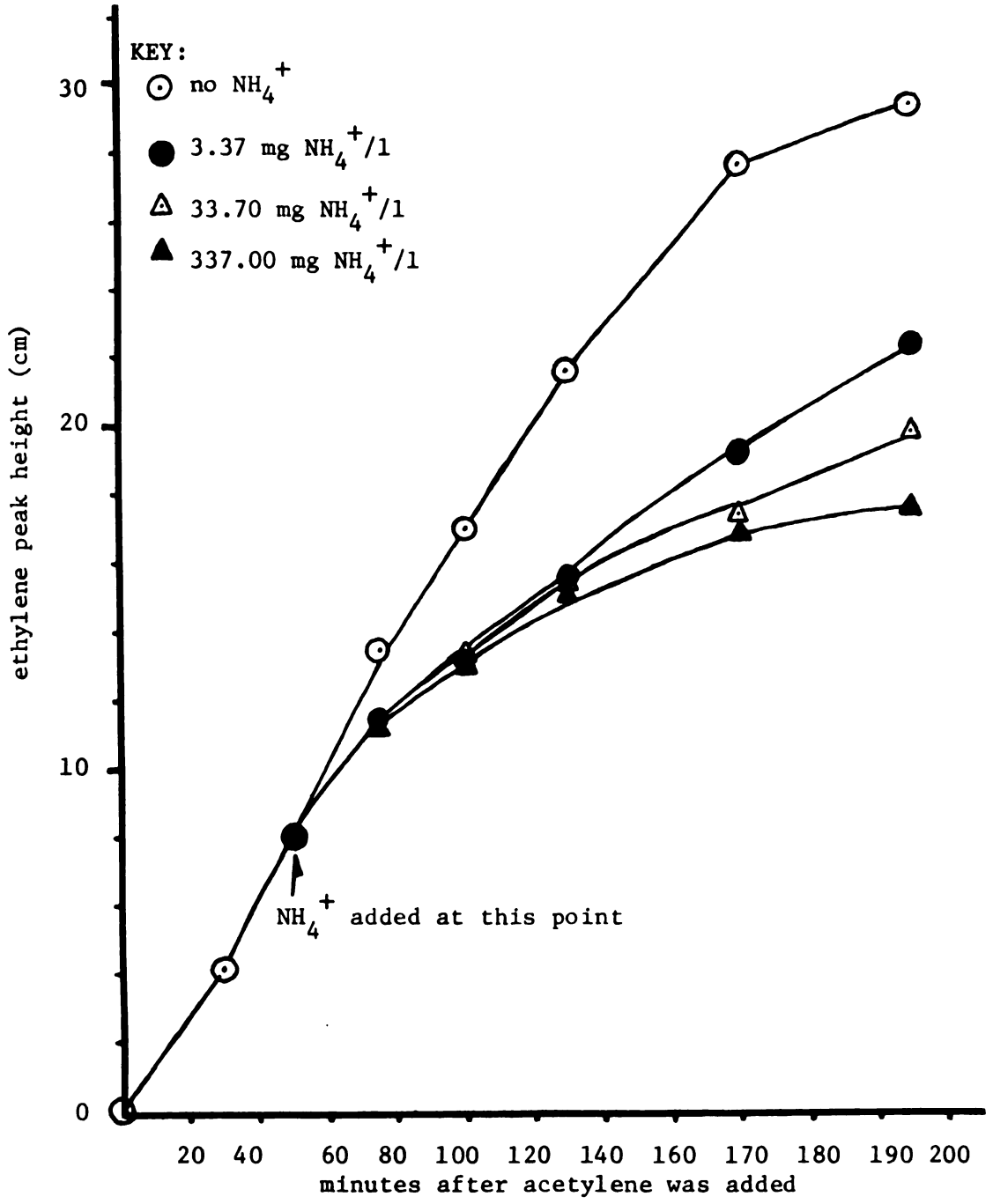


Figure 10. The effect of NH_4^+ on ethylene production by *Azotobacter vinelandii* with time.



Nitrogen fixation during the growth of *Azotobacter vinelandii* in laboratory cultures was studied (Figure 11). Ethylene production begins early in the exponential phase and increases until about the middle of the exponential phase, at which time the amount of ethylene produced per cell begins to decrease. The amount of ethylene produced reaches zero at the beginning of the stationary phase.

Ethylene production during the growth of the *Azotobacter chroococcum* strain isolated from the glucose enrichment was studied. When the rate of ethylene production approached zero, cells in one flask were centrifuged, washed with sterile water, and resuspended in fresh medium. The absorbance continued to increase while ethylene production decreased (Figure 12).

Cyst formation was induced in *Azotobacter vinelandii* by 0.2% β-hydroxybutyric acid. Nitrogen fixation during induced encystment decreased to an almost undetectable amount 1 hour after introduction into 0.2% BHB (Figure 13).

Most probable numbers of *Azotobacter* in Wintergreen Lake were determined in July 1971 using an MPN index. The results (Table 7) indicate that there is 1 *Azotobacter* cell in 2 ml of lake water.

Two isolates with *Azotobacter* morphology were obtained from enrichment cultures of a mud-water interface sample from Wintergreen Lake. The one strain that grew in ethanol produced yellow, raised, circular, gummy colonies on solid medium plus 1% glucose. The other isolate, from the glucose medium, produced slimy, spreading colonies which turned brown with age. The results of the utilization by *Azotobacter* of various carbohydrates are presented in Table 8. *Azotobacter vinelandii*, *A. chroococcum*, and both isolates formed cysts on

Figure 11. Ethylene production during growth of *Azotobacter vinelandii* in Burk's buffer plus 1% glucose.

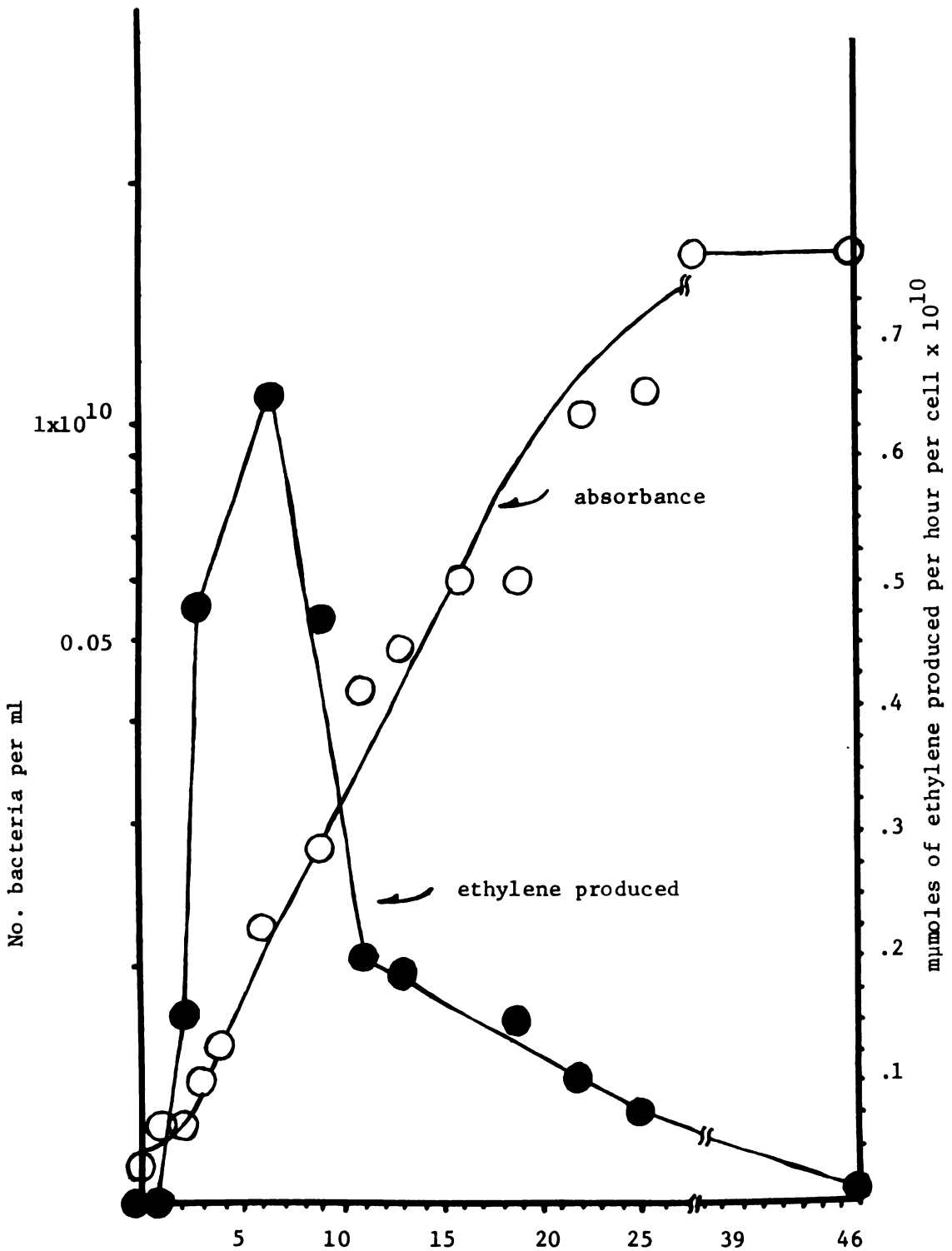


Figure 12. The effect of fresh medium on ethylene produced.

Key:

- ○ absorbance of control culture
- ● ethylene produced in control culture
- □ absorbance in culture resuspended in fresh medium at 17 hr
- ◪ ethylene produced in culture resuspended in fresh medium at 17 hr
- △ absorbance in culture resuspended in fresh medium at 23 hr
- ▲ ethylene produced in culture resuspended in fresh medium at 23 hr

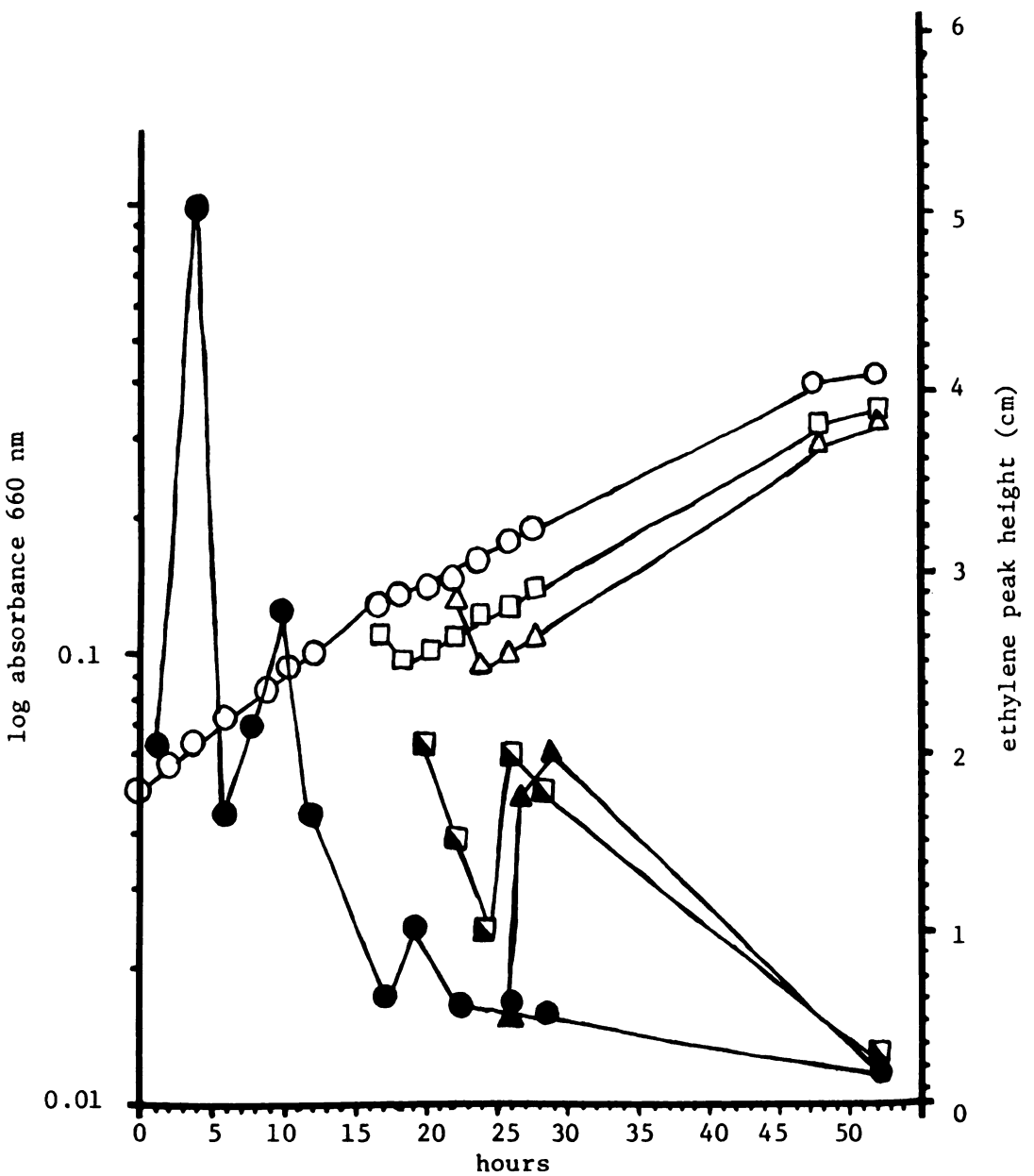


Figure 13. N_2 fixation during encystment

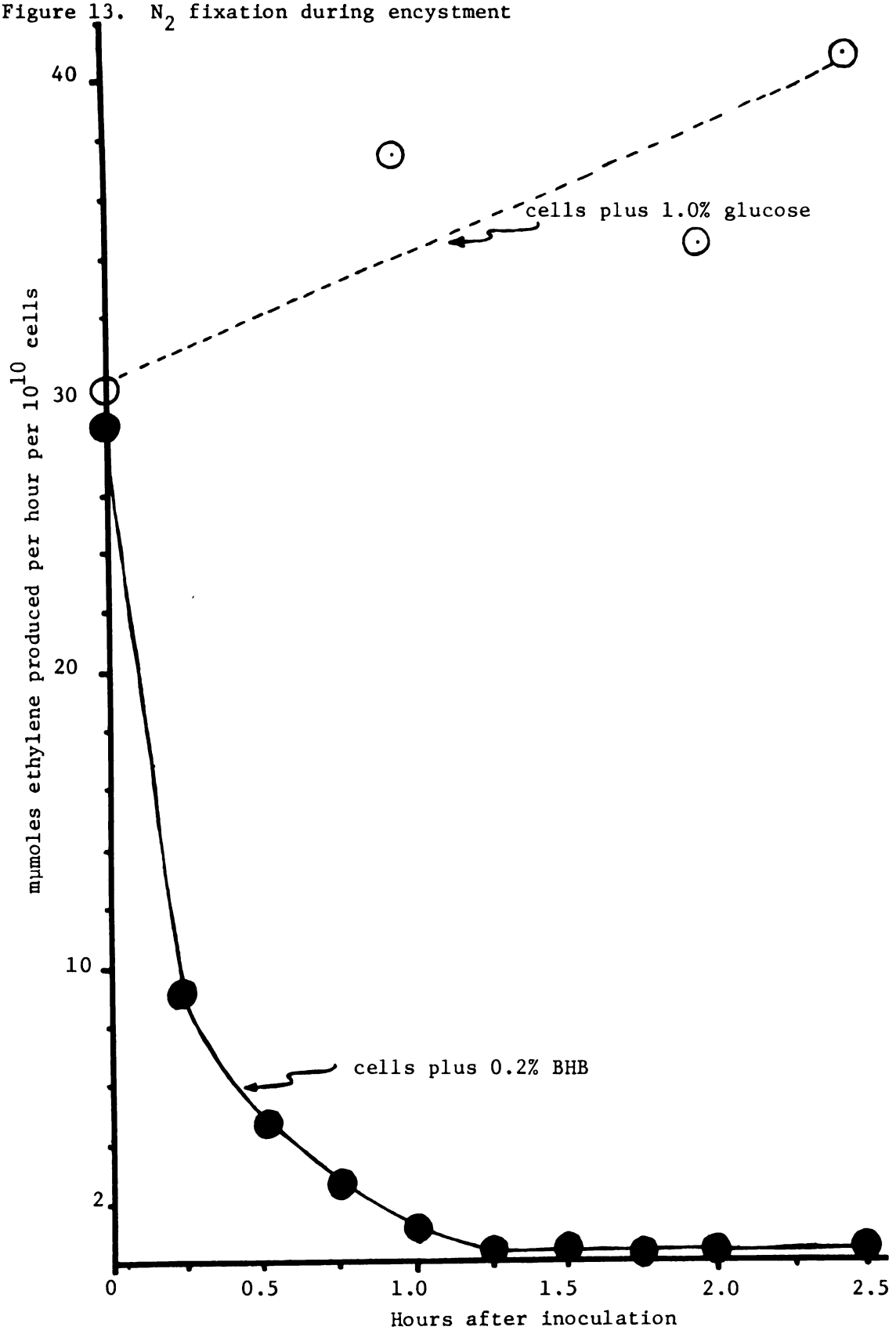


Table 7. Most probable numbers of *Azotobacter* in Wintergreen Lake

	No. of tubes giving positive reactions out of 5 tubes					Combination of positives	MPN index per 100 ml
dilution	10^0	10^1	10^2	10^3	10^4		
	2	0	0	0	0	2-0-0	50

Table 8. Utilization of various carbon sources by *Azotobacter*

Carbon source	*A. vinelandii*	*A. chroococcum*	*A. agilis*	Isolate from glucose	Isolate from ethanol
mannitol	+4	±	±		±
ethylene glycol	+1	±	±		±
resorcinol	+1				
butanol	+3	+2		+3	+3
starch	+4	+4	±	+4	+2

Growth was scored as +1 to +4 with ± being barely visible growth.

butanol. Both isolates appeared to be *A. chroococcum*. Figures 14 and 15 show the pattern of ethylene production during growth of the isolated strains of *Azotobacter*. Ethylene production begins in the late lag phase and increases until about the middle of the exponential phase, at which time the amount of ethylene produced begins to decrease.

The 2 isolates of *A. chroococcum* from Wintergreen Lake were tested for growth and acetylene reduction under various partial pressures of oxygen. The *A. chroococcum* strain isolated on glucose grew equally well in 20, 5, and 0.95% oxygen atmospheres (Figure 16). The amount of ethylene produced increased in the samples grown in the 0.95% and 0.1% oxygen atmospheres (Table 9). The strain isolated on ethanol showed a different pattern of growth. It grew best under an atmosphere of 5.0% oxygen (Figure 17). Ethylene produced increased in the 20% and 0.1% oxygen atmospheres (Table 9).

Table 9. Acetylene reduction during the growth of 2 strains of *Azotobacter chroococcum* under various partial pressures of oxygen

	μmoles ethylene/absorbance unit 660 nm			
	Azotobacter chroococcum isolated on glucose		*Azotobacter chroococcum* isolated on ethanol	
% oxygen	24 hr	33 hr	24 hr	33 hr
20	0.316	0.037	0.328	0.415
5	0.045	0.037	0.037	0.031
0.95	0.015	0.030	0.030	0.030
0.1	0.033	0.035	0.028	0.031

Figure 14. N_2 fixation during growth of *Azotobacter chroococcum* strain 1 isolated from an ethanol enrichment.

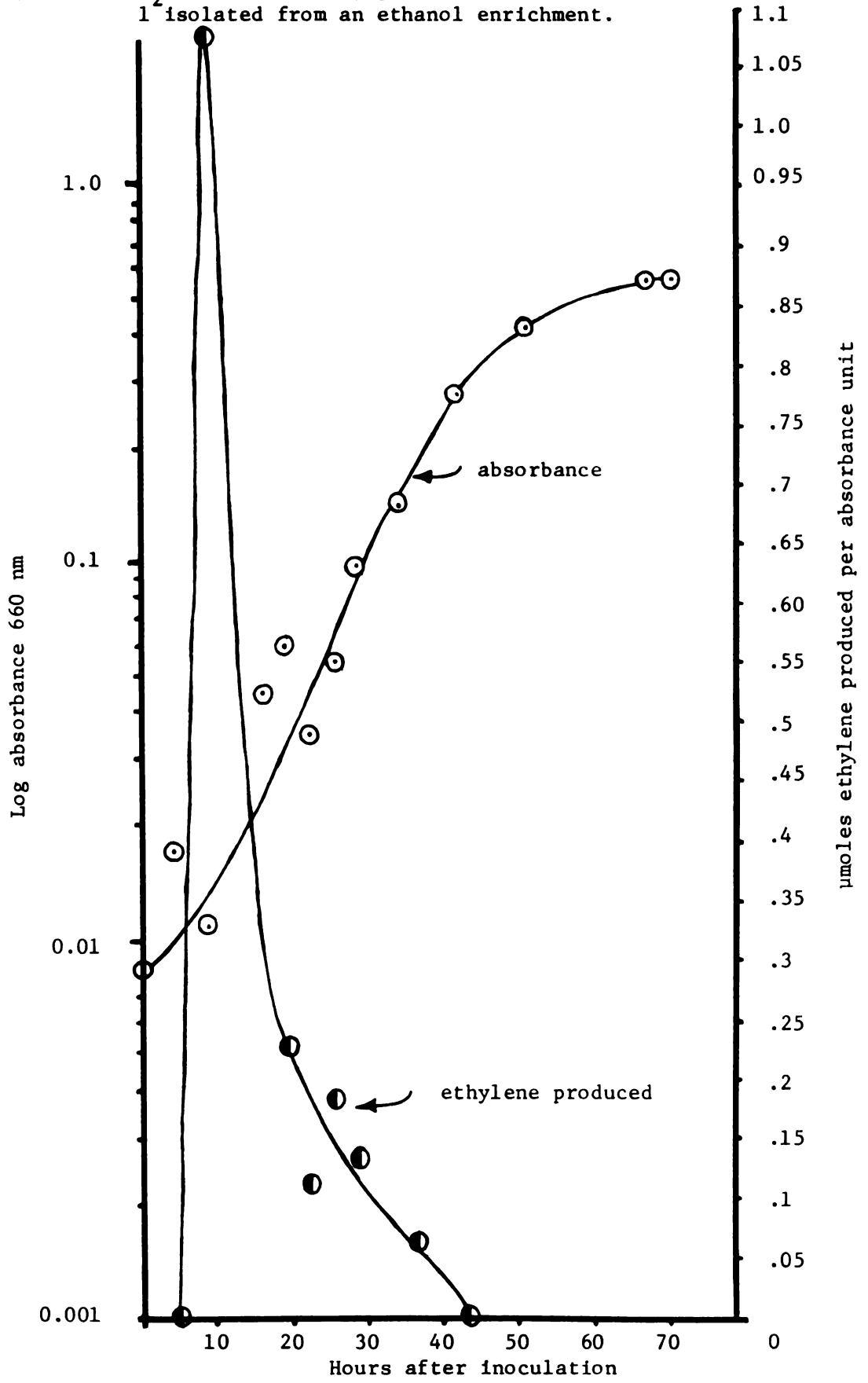


Figure 15. N_2 fixation during growth of *Azotobacter chroococcum* strain 2 isolated from a glucose enrichment.

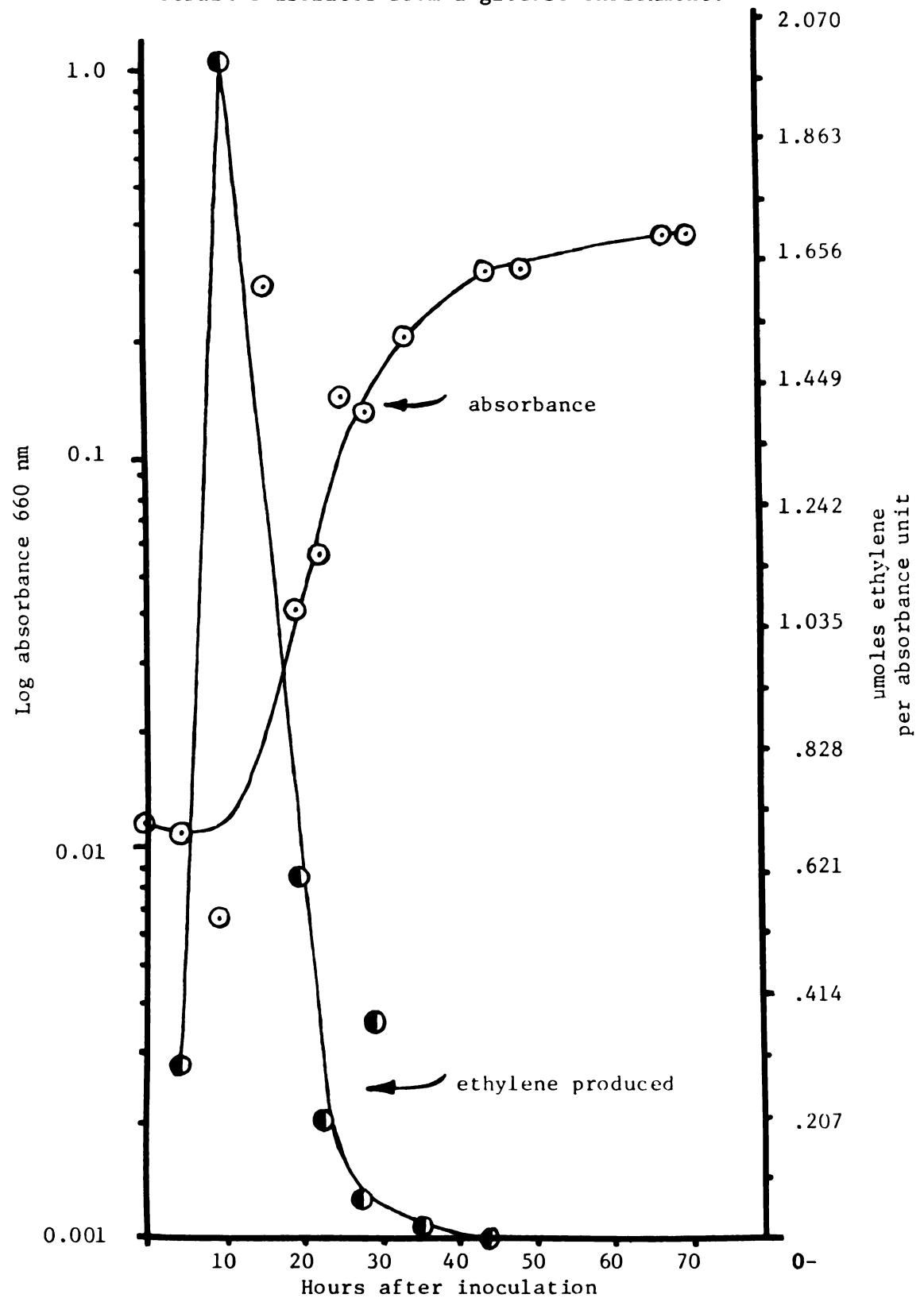


Figure 16. Growth of *Azotobacter chroococcum* isolated on glucose under various partial pressures of oxygen.

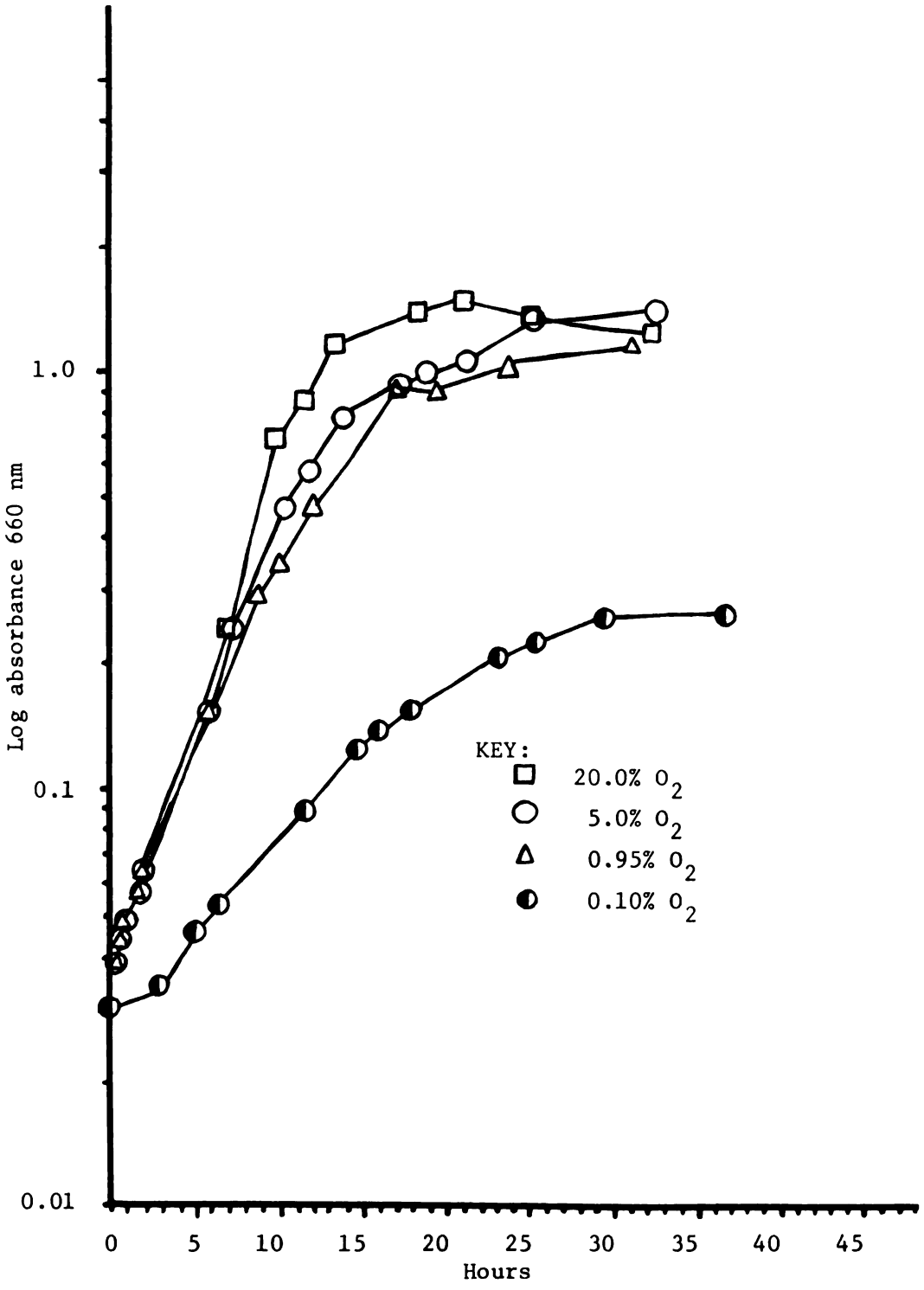


Figure 17. Growth of *Azotobacter chroococcum* isolated on ethanol under various partial pressure of oxygen.

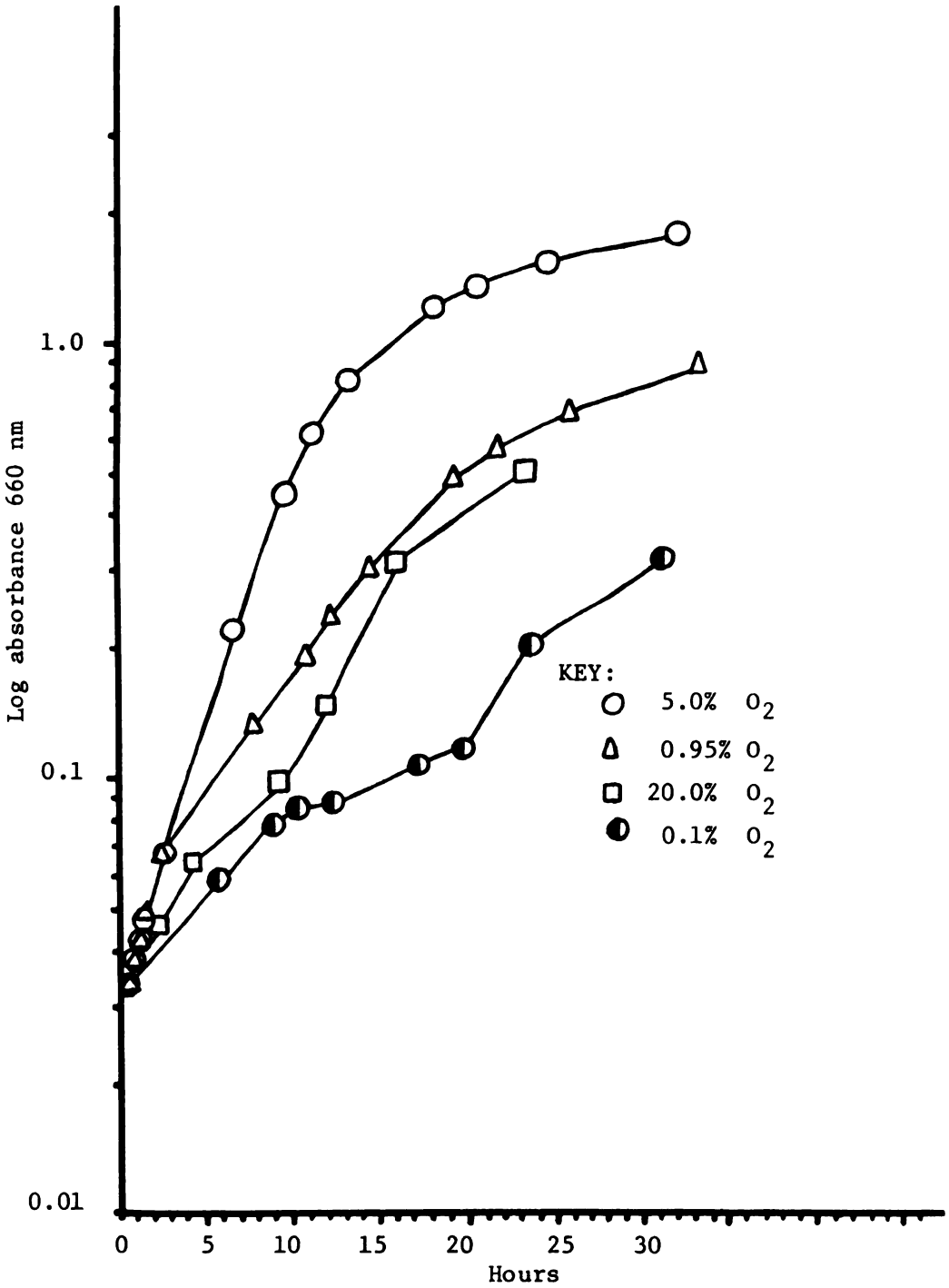
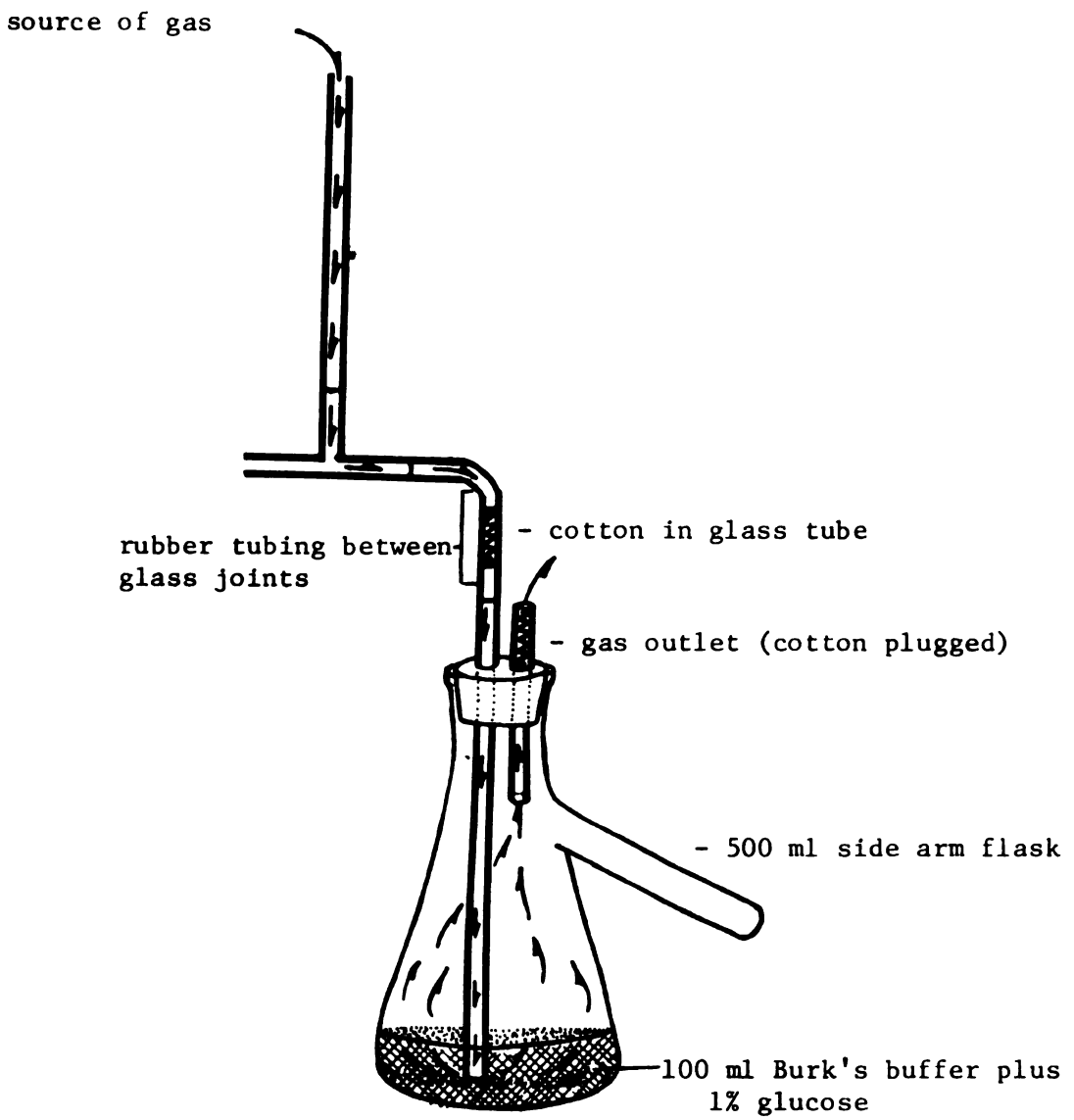


Figure 18. Gas flow apparatus used in studies of growth under various partial pressures of oxygen.



DISCUSSION

Studies of cell-free extracts from various N_2-fixing systems have shown that the N_2-fixing enzyme, nitrogenase, catalyzes the reduction of acetylene to ethylene (14,20,40). The reduction of N_2 to $2NH_3$ requires the transfer of 6 electrons, whereas the reduction of acetylene to ethylene requires 2 electrons. Studies on the correlation between acetylene reduction and nitrogen fixation indicate that the ratio of moles of N_2 fixed to moles of ethylene produced ranges from 3 to 4.5 (20). Stewart, Fitzgerald, and Burris (43) have shown that it is feasible to use the rate of ethylene production as an index of the rate of nitrogen fixation. This indirect technique has several advantages over the conventional ^{15}N technique. It is: (a) relatively inexpensive, (b) easier, (c) a more sensitive method, and (d) short-term exposures allow measurements of short-term changes.

Nitrogen is a competitive inhibitor of acetylene reduction (14). Flushing with a gas phase containing no nitrogen upsets the equilibrium of dissolved gases causing N_2 dissolved in the liquid to come out of solution. The importance of flushing can be seen in Table 1; flushing causes a threefold increase in ethylene production.

The main objective of this study was to determine if *Azotobacter* fixed nitrogen in Wintergreen Lake. Studies of nitrogen fixation by *Azotobacter vinelandii* indicated that nitrogen is not being fixed either in Wintergreen Lake water or Burk's nitrogen-free buffer until a minimum of 100 µg glucose per milliliter is added (Table 2). This indicated

that an insufficient concentration of an energy source may be limiting the rate of nitrogen fixation. However, even when glucose was added, the amount of ethylene produced in lake water compared to that in Burk's buffer appeared to be only about one-half the maximum amount found for the buffer. The amount of ethylene produced in Burk's buffer plus 10,000 μg glucose per milliliter was considered maximal, because optimal concentrations of all elements essential for growth are present. These results indicated that the cells were capable of reducing acetylene but that something other than a carbon source was affecting the amount of nitrogen fixed. The presence of a combined nitrogen source or a difference in pH might have affected the amount of nitrogen fixed.

Acetylene reduction was observed only in the sample containing Burk's buffer plus 1% glucose when the experiment was done in the lake. The general methods used were the same; however, the lake water used in the laboratory studies and in the lake studies was collected at different times, July and October, respectively. The lake water was collected, filtered, and stored at 4 C for use in laboratory studies, whereas in the lake experiment water was collected, filtered, and immediately used. The chemical composition of the lake varies with the time of year, levels of NH_4^+ show an annual range of 0.005 to 2.32 mg/l, NO_2^- ranges from 0.012 to 0.04 mg/l, and NO_3^- ranges from 0.005 to 1.34 mg/l (33). The levels of combined nitrogen are low from June to August and high from September to April (33). The temperature of the lake also varies. Laboratory experiments were conducted at 25 C (the temperature of the surface of the lake in midsummer), and the lake experiment was done at 15 C. The effects of temperature on nitrogen fixation have not been studied; however, 15 C is near the

minimum temperature for growth (26), and the amount of nitrogen fixed has been shown to be proportional to the amount of growth.

The results of experiments on nitrogen fixation in lake water indicate that *Azotobacter vinelandii* does not fix nitrogen in Wintergreen Lake. There are several explanations for the absence of nitrogen fixation in lake water. The prime explanation is that an available energy source is lacking in lake water. *Azotobacter vinelandii* was suspended in lake water to determine if it remained viable and grew. Studies on the viability of *Azotobacter vinelandii* in Wintergreen Lake water indicate that, although cells transferred to either lake water or Burk's buffer show an initial increase in cell number, the cells do not actively grow in the lake water (Table 3). The early growth could be due to the carryover of some glucose when the cells were transferred, to the utilization of storage products, or to the cells being ready to divide at the time of transfer. Because of this, the calculation of generation time of *Azotobacter vinelandii* in lake water and in Burk's buffer was based on the 12- to 48-hr interval after resuspension when the increase of cell number in Burk's buffer was not so rapid. The generation times, 29.3 hr in lake water and 27.3 hr in Burk's buffer, are not appreciably different indicating maintenance level activity. The generation time of cells growing in Burk's buffer plus 1% glucose was 5.4 hr. This is approximately one-fifth that in lake water not supplemented with glucose.

Studies with cells grown in medium containing labeled glucose indicate that the cells respire in the lake water at about the same rate as they do in Burk's buffer, indicating maintenance-level respiration (Table 4). Some ^{14}C-glucose was carried over in the supernatant solution, and this was rapidly depleted to a constant level after 2

hours (Figure 2). The $^{14}CO_2$ produced after 2 hours then indicated respiration in the new medium. The Burk's buffer is essentially a starvation medium. The respiration rate during the 16-hr interval from 2 to 18 hours after the cells were resuspended in lake water was 1.4×10^{-7} µmoles $^{14}CO_2$/hr compared to 1.3×10^{-7} µmoles $^{14}CO_2$/hr in Burk's buffer. It must be noted that the pH of the lake water was 8.5, whereas that of the Burk's buffer was 6.8. At pH 8.5 dissolved carbon dioxide tends to be in the bicarbonate form. At pH 6.8 more tends to be released from solution as CO_2; however, significant amounts of dissolved CO_2 will not be released from solution until pH 6.3. To have an accurate measure of CO_2 produced by the cells, dissolved CO_2 should be driven from the solution by the addition of acid. The fact that this was not done should not alter the results. Slightly more $^{14}CO_2$ was trapped in KOH in the lake water sample than in the buffer sample. Since more CO_2 would be dissolved in lake water at pH 8.5 than in the buffer at pH 6.5, the addition of acid would have only enhanced the amount of $^{14}CO_2$ trapped in the lake water sample. This would still remain above that observed in the buffer sample. By continually removing CO_2 in the KOH trap, the equilibrium is forced toward CO_2 formation.

If the cells release 1.4×10^{-7} µmoles of CO_2/hr/10^{10} cells in lake water, the amount of energy available to the cells can be calculated. In the citric acid cycle, 38 ATP molecules are produced from 1 molecule of glucose which is completely oxidized to 6 CO_2 and water. This means that if 1.4×10^{-7} µmoles of CO_2 were detected, 8.74×10^{-7} µmoles of ATP were produced. Four ATP are required for the transfer of one electron pair. This means that 6 µmoles of ATP are required per µmole NH_3 produced, or 4 µmoles ATP are required per µmole of ethylene produced.

If the entire amount of ATP produced were to be used in nitrogen fixation, the maximum amount of NH_3 formed would be 1.46×10^{-7} µmoles; or, in the acetylene reduction technique, 2.19×10^{-7} µmoles of ethylene would be formed. These low quantities of NH_3 or ethylene indicate that the cells do not have the energy required for nitrogen fixation. It should be noted that since acid was not added to the cell suspension to drive off dissolved CO_2, the estimate of CO_2 evolved may be low.

The protein-synthesizing system was still active in cells suspended in lake water (Figure 3). That the cells exhibited a greater rate of protein synthesis in lake water was indicated by greater incorporation of L-leucine-U-^{14}C into cold TCA-insoluble precipitates. Since leucine is one of the common amino acids found in protein, it is probably immediately incorporated into leucine tRNA which then is used in protein synthesis. The rate of leucine incorporation was stimulated by the addition of 2 mg glucose/ml of lake water or Burk's buffer. However, the rate of protein synthesis in lake water remains greater than in Burk's buffer whether or not glucose was added. This indicates that there was something in lake water which promoted leucine incorporation; the lake water does have some available energy sources and a source of nitrogen NH_4, NO_2, and NO_3 (33), which the buffer does not have.

An attempt was made to determine the amount of glucose in lake water using the kinetic analysis of Hobbie and Wright (49) of ^{14}C-glucose uptake by a natural population. The kinetic analysis is based on Michaelis-Menten kinetics. When uptake of a solute is mediated by a transport system located at the cell membrane, the rate of uptake is describable in terms of Michaelis-Menten kinetics (25).

According to Wright and Hobbie (50), the measurable uptake of glucose at close to natural levels of concentration always follows Michaelis-Menten kinetics. The original equation derived by Michaelis and Menten is:

$$v = \frac{V(S)}{K_m + S} \qquad (A)$$

In terms of uptake transport systems, v is the velocity at a given substrate concentration S; V is the maximum velocity attained when substrate saturation of the uptake sites occurs; K_m, the Michaelis constant, is by definition the substrate concentration when the velocity v is exactly one-half the maximum velocity V. To avoid confusion, the Michaelis constant is called K_t, the transport constant.

Equation (A) may be rearranged to give the Lineweaver-Burk equation, derived by taking the inverse of equation (A) and multiplying both sides by S:

$$S/v = K_t/V + S/V \qquad (B)$$

By plotting S/v against S, a straight line is usually obtained. The linearity of the plot reflects how well the increase in velocity of the reaction in question corresponds to substrate concentration in the manner required by the Michaelis-Menten equation. Once one knows if the system follows Michaelis-Menten kinetics, the Lineweaver-Burk plot can be used to evaluate V_{max} and K_t. The slope is 1/V, the intercept is K_t/V, and the extrapolated line intercepts the abscissa at $-K_t$.

When using radioactive material to measure the uptake kinetics, the velocity of uptake v (mg liter^{-1} hr^{-1}) at a given substrate concentration is given by the formula from Parsons and Strickland (37):

$$v = cf(S_n + A)/C\mu t \qquad (C)$$

In this equation, c is the radioactivity of the filtered organisms (counts/min); S_n is the concentration (mg/liter) of a given substrate present in the natural sample; A the concentration (mg/liter) of added substrate; C the count/min from 1 μc ^{14}C in the counting assembly used; μ the number of microcuries added to the sample bottle; and t is the incubation time (hr). In this present analysis f, a factor to correct for isotope discrimination, is neglected because it is unknown.

Equations (B) and (C) may be combined in an equation that describes the uptake kinetics of natural plankton when the natural substrate is unknown (49). The substrate concentration S in equation (B) is really the unknown plus the added substrate, or $(S_n + A)$. Equation (C) is rearranged so that:

$$(S_n + A)/v = C\mu t/c \qquad (D)$$

This, substituted into equation (B) gives

$$C\mu t/c = (K_t + S_n)/V + A/V \qquad (E)$$

With this equation, the data from uptake measurements from plankton at several low substrate concentrations are plotted as Cμt/c versus [A] (Figure 4), giving the values for $(K_t + S_n)$ and V.

The intercept of the plot is actually a measure of the turnover time for the substrate due to uptake by means of a transport system (2). This intercept, obtained by extrapolation, is the point where A = 0. From equations (D) and (E):

$$C\mu t/c = S_n/v_t = (K_t + S_n)/V = T_t \qquad (F)$$

T_t is the time (hr) required for the substrate to be entirely removed by the natural population. A constant rate of removal and continual regeneration are assumed.

This indirect method for determining natural substrate concentration is dependent on 4 assumptions: (a) that isotopic discrimination is minimal, (b) that the amount of C-14 excreted is insignificant, (c) that C-14 is actually being incorporated into macromolecules subsequent to uptake, and (d) that the natural substrate concentration is much smaller than the concentration of added substrate. The determination of natural substrate concentration is based on extrapolation which may not be valid. The method is based on the uptake and not growth; consequently, the C-14 glucose may be taken into the cell and stored, it may be incorporated into macromolecules, it may be respired and lost as $^{14}CO_2$, or it may be adsorbed to cell surfaces. The natural population of organisms may respond to the added glucose by a switch in enzyme activity. Catabolite repression may occur; the increase in the concentration of glucose could shut off the uptake of other carbon sources and show an increase in the uptake of glucose. This would affect the observed velocity of uptake and slope of the line which is extrapolated to obtain the concentration of natural substrate in the lake. An unlabeled control cannot be used.

The results (Figure 4) indicate that the system follows Michaelis-Menten kinetics. The maximum level of glucose present in the lake is calculated to be 13.5 µg/liter. This is not enough to promote growth and nitrogen fixation by *Azotobacter* (see studies on viability and nitrogen fixation in lake water). Glucose is not the only carbon source in the lake. Manny (33) reports that the annual range of dissolved carbon in Wintergreen Lake varies from 6 to 9.1 mg l^{-1} at a

1 m depth. This dissolved carbon could include acetate, peptides, and organic acids.

We know from the studies of viability, respiration, and protein synthesis in lake water that the cells have essentially a maintenance level metabolism. Suitable energy sources appear to be limiting. Because nitrogen fixation requires energy, and the energy is not available, nitrogen fixation is not detected.

When glucose was added to lake water, the cells were able to reduce acetylene. However, even when glucose was added, the amount of ethylene produced in lake water compared to that in Burk's buffer appeared only about one-half the maximum amount found for the buffer. This indicated that something, other than an energy source, was affecting the amount of nitrogen fixed. It has been shown that combined nitrogen represses nitrogen fixation in *Azotobacter* (8,10,21,39). Vukhur and Iwasaki (39) report that ammonia nitrogen at a concentration as low as 410 mg ammonium per liter will inhibit nitrogen fixation in a synthesis medium. Using the sensitive acetylene reduction technique, it appears that as little as 26 mg ammonia nitrogen per liter will inhibit nitrogen fixation in Burk's buffer plus 1% glucose (Figure 9). Ethylene production with time gradually decreases when ammonium is added to a cell suspension producing ethylene (Figure 10). It is postulated that the ammonium prevents the synthesis of nitrogenase, and the existing nitrogenase was diluted out (42). Studies of the ammonia concentration in Wintergreen Lake (33) indicate that ammonia concentrations have an annual range of 0.005 to 2.32 mg/liter at a 1 m depth. Since the surface waters are fairly well mixed, the concentration in the surface layers should be uniform. There were also detectable amounts of nitrite and nitrate in the lake water (33).

Nitrite and nitrate will also be utilized by *Azotobacter* in preference to atmospheric nitrogen (10,21,39). The amounts of ammonia, nitrite, and nitrate in the lake water fluctuated throughout the year. They were lowest from June through August, a time when there was an increased number of organisms which utilize the combined nitrogen for growth (33). Since the laboratory experiments on nitrogen fixation in lake water were done in July, it is highly probable that the levels of combined nitrogen in the lake water were low, NH_4 0.005 mg/l, NO_2 0.012 mg/l, NO_3 0.005 mg/l (33). In October, the combined nitrogen levels are high; ammonia concentration may be as high as 2.32 mg/l, which might account for why no nitrogen was fixed by cells suspended in lake water at that time.

Studies by Oppenheim and Marcus (35) on the correlation of the ultrastructure of *Azotobacter vinelandii* with nitrogen source for growth indicated that *Azotobacter* synthesizes an extensive, internal, membranous network when grown under conditions requiring nitrogen fixation. When *Azotobacter* grows with fixed nitrogen, i.e., ammonia or amino acids, very slight quantities of internal membrane were found. These were concentrated mainly about the cell periphery. A correlation between the ultrastructure of *Azotobacter* with nitrogen fixation would have been a useful tool in indicating the capacity of *Azotobacter* to fix nitrogen. When cells were grown with N_2 and ammonia for nitrogen sources, the same internal membrane network was visible in all cells (Figures 5 through 8) regardless of the nitrogen source in the medium. These results differed from those obtained by Oppenheim and Marcus. There were 2 basic differences in the procedure: the cells were not aerated on a shaker, and the cells were double fixed with glutaraldehyde and osmium tetroxide. The double fixation process

allows better preservation than with simple fixation in OsO_4. Therefore, it seems probable that the differences in the internal membrane network observed by Oppenheim and Marcus was due to the aeration rather than to the nitrogen source for growth. The ultrastructure of the cell does not appear to be a good indicator of whether the cells are fixing nitrogen or not.

The difference in pH between lake water, pH 8.5, and Burk's buffer, pH 6.8, may make a difference in the amount of nitrogen fixation observed; the nitrogenase enzyme operates over a fairly narrow pH range near pH 7.0, with fixation falling off markedly above and below pH 6.5-7.0 (9). To see if the pH does affect nitrogen fixation, the pH of the Burk's buffer should be adjusted to that of the lake water and the nitrogen fixation tested.

The pattern of acetylene reduction during growth of cells in a normal laboratory culture was studied. Ethylene production began in the late lag phase or early exponential phase of growth. Ethylene production increased until about the middle of the exponential phase, at which time the amount of ethylene produced per cell began to decrease (Figures 11, 14 and 15). One would expect that ethylene production would continue throughout the exponential stage at the same rate as the cells were actively metabolizing and in need of nitrogen. The possibility existed that the cells accumulated waste products in the medium would inhibit the process; accumulation of nitrogen inside the cell would show the same inhibition. When the rate of ethylene production in a culture approached zero, cells were washed and resuspended in fresh medium. The absorbance continued to increase while the ethylene production decreased (Figure 12). This indicated that the decrease in the ethylene production was not due to waste product

buildup but, rather, to the physiological state of the cell. The culture is nonsynchronous; the cells are not all in the same physiological state. For example, cysts may have been present in the late exponential phase.

Cyst formation in *Azotobacter* is induced by 0.02% β-hydroxybutyric acid (30). Studies of nitrogen fixation during induced cyst formation indicate that nitrogen fixation rapidly decreases to an almost undetectable amount 1 hour after introduction into 0.2% BHB (Figure 13). This indicates that cysts do not fix nitrogen. Since BHB is not normally found in the natural environment, the cyst, induced by BHB, may be a laboratory phenomenon. Layne and Johnson (29) indicate that cysts may be induced by a lack of or suboptimal concentration of several minerals. Cysts formed under mineral deprivation do not have the typical ultrastructure of cysts formed in BHB. Nitrogen fixation by cysts formed under these conditions has not been investigated. Mineral deficiency in the natural environment might cause cysts to be formed.

Two isolates with *Azotobacter* morphology were obtained from Wintergreen Lake. The morphology, carbon utilization, acetylene reduction, pigment and cyst production indicated that the isolates are both strains of *Azotobacter chroococcum* according to the classification of *Azotobacter* by Norris and Chapman (34) and Voets and Dedeken (45). The pattern of ethylene production during growth was the same for them as that observed for *A. vinelandii*. The sample from which the two strains of *A. chroococcum* were isolated was not obtained aseptically introducing the possibility that the organisms were not indigenous to the lake. The isolates did not grow as rapidly or as well as *A. vinelandii* in the Burk's nitrogen-free buffer plus 1% glucose.

Therefore, *A. vinelandii* was used in the major studies on nitrogen fixation.

The isolates of *Azotobacter vinelandii* were tested for growth and acetylene reduction under various partial pressures of oxygen. The results (Figures 16 and 17) indicated that the 2 strains had different oxygen requirements. The *A. chroococcum* strain isolated on glucose grew equally well in 20, 5, and 0.95% oxygen atmospheres. The strain isolated on ethanol grew best under an atmosphere of 5.0% oxygen. The ethylene produced (Table 9) was only measured at 2 points and may not be a valid indication of what actually occurred.

These studies indicate that *Azotobacter vinelandii* does not significantly contribute to nitrogen fixation in Wintergreen Lake water. It does not fix nitrogen in lake water. Energy sources appear to be limiting. *Azotobacter vinelandii* exhibits a very low metabolic rate, low respiration rate and a low growth rate in lake water. Most probable number studies (Table 8) indicated that *Azotobacter* are present in very low numbers in the lake, about 1 organism per 2 milliliters of lake water. This is only an indication of the number of azotobacters of any species. It is important to note that the *Azotobacter vinelandii* used in these studies was not indigenous to the lake.

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APPENDIX

APPENDIX

A Sterile Sampling Device for Lake Water and a Holder for Incubating Samples in the Lake

Samples were obtained aseptically in sterile eye and ear syringes. A pasteur pipette was sealed at one end. The open end was inserted into an eye and ear syringe that had the air forced out of it. This caused a vacuum. The sampling device was wrapped in aluminum foil and autoclaved. At the lake site, these were carefully unwrapped and placed in a wire basket suspended by a plastic cord and weighted down with a weight. A small wire ring was placed around the pasteur pipette to hold it upright. The sampler was lowered to the desired depth and a weight dropped down the plastic cord. This broke the pasteur pipette, and water was drawn into the bulb (Figure 19).

A plexiglass holder was designed and built to maintain 16 serum bottles at a single depth in the lake. The holder can be lowered and maintained at any depth by adjusting the nylon cords which attach to a buoy. The holder prevents sample bottles from tilting and holds them tightly so they cannot be lost. The holder fits easily on the lap, forming a workbench during inoculation and addition of various chemical substances (Figure 20).

Figure 19. A sterile sampling device for lake water.

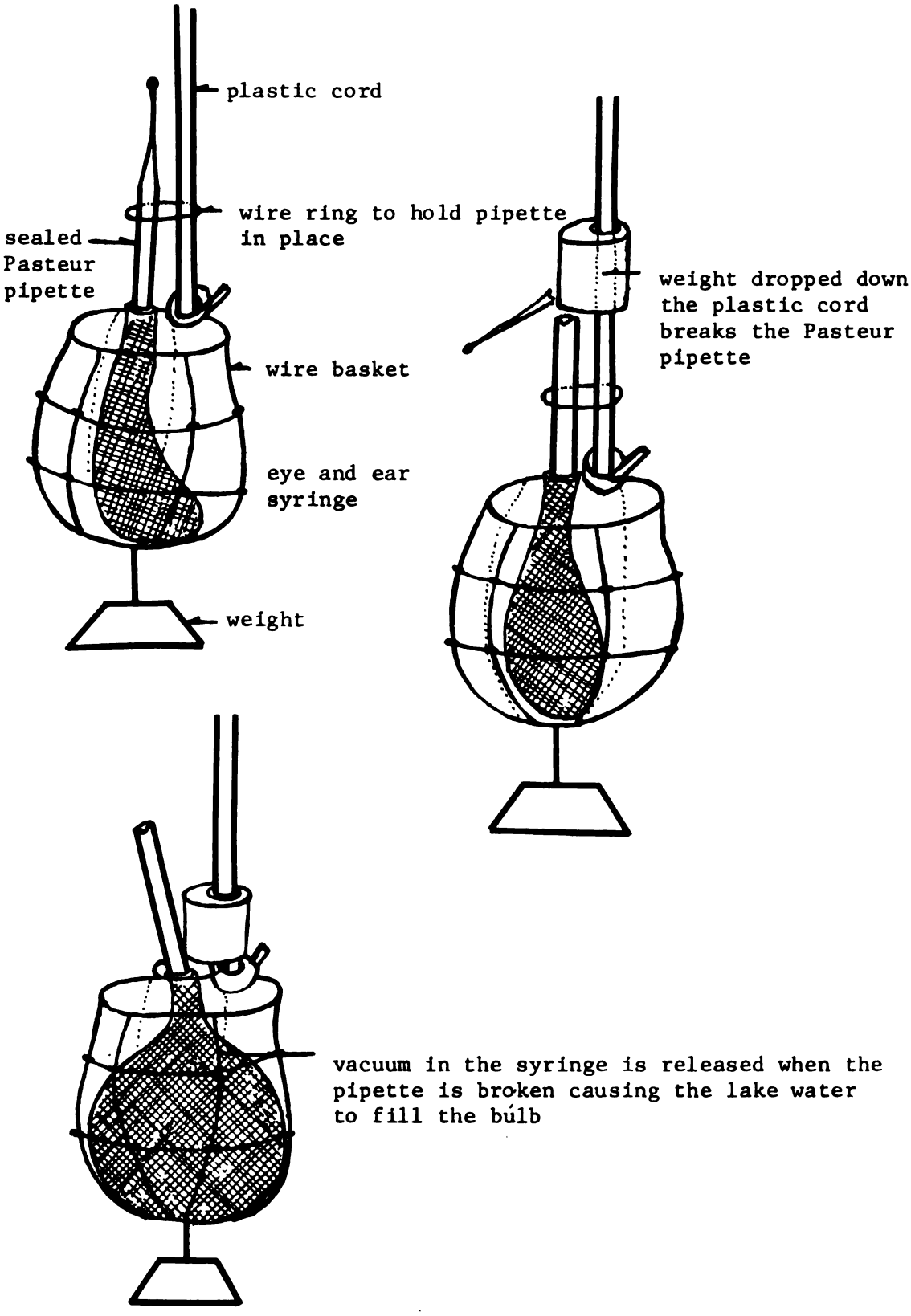

Figure 20. Plexiglass holder for incubating samples in the lake.