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PHYSIOLOGY OF NITROGEN FIXATION BY  
A SPECIES OF ACHROMOBACTER

BY

ROYAL DON GCERZ

A thesis submitted  
in partial fulfillment of the requirements for the  
degree Master of Science, Department of  
Bacteriology, South Dakota State  
College of Agriculture  
and Mechanic Arts

August, 1960

PHYSIOLOGY OF NITROGEN FIXATION BY

A SPECIES OF ACHROMOBACTER

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

\_\_\_\_\_  
Thesis Advisor

\_\_\_\_\_  
Head of the Major Department

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RDG

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## INTRODUCTION

The metabolic pathway by which certain microorganisms incorporate atmospheric nitrogen into their protoplasm is at the present time unknown. In an attempt to determine this pathway scientists are studying the physiology of various nitrogen fixing bacteria and are within reasonable limits extrapolating this information from one organism to another. Thus far they have found certain physiological characteristics which appear to be common to all nitrogen fixing bacteria.

Several years ago a species of the genus Achromobacter was isolated and shown to fix atmospheric nitrogen. Thus far very little has been published concerning the physiology of nitrogen fixation by this organism. This study was carried out in an attempt to confirm some of the reported findings regarding the physiology of nitrogen fixation by this organism and to determine other physiological characteristics which may be of value in the elucidation of the mechanisms of biological nitrogen fixation.



## REVIEW OF THE LITERATURE

Relatively few organisms are known to be able to use atmospheric nitrogen as their nitrogen source. These are reviewed by Wilson (17). Since Wilson's review several papers have appeared indicating that other bacteria are able to fix nitrogen. These include Bacillus polymyxa (5), one strain of Achromobacter (7), and several strains of Pseudomonas (1, 13).

Oxygen has been shown to be a specific inhibitor of nitrogen fixation in many organisms investigated including the obligate aerobe, Azotobacter vinelandii (8). Parker and Scott (8) review the literature concerning the effect of oxygen on nitrogen fixation by various organisms, but do not include any discussion of the effect of oxygen on nitrogen fixation by the present strain of Achromobacter. Jensen (7) reported that this strain, which he designated N4, will fix nitrogen either anaerobically or aerobically. He found, however, that oxygen had a clearly depressing effect. Proctor and Wilson (14) refer to this strain as N7 and also report that it is able to fix nitrogen anaerobically as well as aerobically. They found that the efficiency of fixation in terms of quantity of nitrogen fixed per milligram of carbohydrate utilized was much greater under anaerobic conditions than it was under aerobic conditions.

Members of the family of leguminous plants and members of the bacterial genus Rhizobium, neither of which can fix nitrogen themselves, are able to enter into a symbiotic relationship and are then able to utilize

atmospheric nitrogen. Wilson and Umbreit (cited in 17) in 1937 discovered that hydrogen is a specific inhibitor of symbiotic nitrogen fixation. Since that discovery, hydrogen has been shown to be a specific inhibitor of nitrogen fixation in Azotobacter (Wyss and Wilson, cited in 17), Clostridium pasteurianum (16), Rhodospirillum rubrum (2), Aerobacter aerogenes (11), and Bacillus polymyxa (4). A preliminary report has also been published concerning hydrogen inhibition of nitrogen fixation by Jensen's strain of Achromobacter (3).

The discovery of hydrogen inhibition of nitrogen fixation in the symbiotic system suggested a possible involvement of hydrogenase in nitrogen fixation. Early attempts to demonstrate hydrogenase in the symbiotic system met with failure (17). Several years later Phelps and Wilson (12) reported that Azotobacter species contained a very active hydrogenase in spite of the fact that they have no apparent need for such an enzyme. This again focused attention on the possible involvement of hydrogenase in nitrogen fixation and scientists began to investigate the nitrogen fixing ability of organisms which were known to possess hydrogenase as a normal part of their hydrogen metabolizing system. As a result of this investigation nitrogen fixation by A. aerogenes and R. rubrum was discovered. Hydrogenase is also known to be present in C. pasteurianum, a well known nitrogen fixing agent, as part of its hydrogen metabolizing system. Recently, hydrogenase has been demonstrated in the symbiotic system (6) and in B. polymyxa (5). The evidence for hydrogenase in Jensen's strain of Achromobacter is still not conclusive. Procter and Wilson (14) have reported that hydrogenase activity was detected when they used the deuterium-hydrogen exchange reaction

of Hoberman and Rittenberg as the method of assay. They were unable, however, to demonstrate hydrogenase in this organism using conventional manometric and Thunberg methods of assay.

## MATERIALS AND METHODS

### Organism

The organism used for this study was a nitrogen fixing species of the genus Achromobacter. This organism, referred to as strain N4, was isolated from a Danish water course and was shown to fix nitrogen by Vagn Jensen (7). It as yet bears no species name. Its physiological characteristics are described by Jensen.

At the beginning of this study physiological tests were made to confirm the identity and purity of the culture, which was carried on nutrient agar containing 0.2 per cent yeast extract.

Because of the fact that growth in most experiments took place under anaerobic conditions in a medium which was essentially free of combined nitrogen the problem of contamination was not great. Periodic checks were made, however, to detect possible contamination by A. aerogenes. This was done by inoculating from experimental cultures into tubes of lactose broth containing gas tubes. The production of acid without the production of gas was used as an indication that the culture was not contaminated with A. aerogenes.

### Medium

Preliminary experiments were carried out using the modification described by Pangra and Wilson (11) of a medium proposed by Monod and Wollman for the cultivation of Escherichia coli. Results of these experiments indicated that Achromobacter N4 produces relatively large quantities of acid during its gasless fermentation of carbohydrate.

Further experimentation revealed that it was possible to increase substantially the quantity of nitrogen fixed by increasing the strength of the phosphate buffer in this medium. Calcium carbonate would not serve as an adequate buffer. In view of the results of these experiments the following medium was devised and used throughout this study:

$\text{Na}_2\text{HPO}_4$ . . . . .	25 grams
$\text{KH}_2\text{PO}_4$ . . . . .	3 grams
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	0.2 grams
$\text{CaCl}_2$ . . . . .	0.01 gram
Sucrose or Mannitol . . . . .	20 grams
Fe-Mb Solution (18) . . . . .	1 milliliter
Distilled Water . . . . .	1 liter

When one or more liters of medium were prepared, the phosphates were dissolved in 70 per cent of the water and the balance of the medium in the remaining 30 per cent. The two solutions were autoclaved separately and combined just prior to inoculation.

For some growth studies and for growing inocula for larger cultures 10 to 30 micrograms of ammonium nitrogen was desired per milliliter of medium. The ammonium was added as the ammonium dihydrogen phosphate salt.

#### Nitrogen determinations

The method used for determination of Kjeldahl nitrogen was essentially the semi-micro method described by Wilson and Knight (18) whereby a small sample was digested, diluted, and treated with Neseler's reagent. Optical density of the treated sample was compared with that of a

standard solution of ammonium sulfate. A Coleman Model 8 Photo-Electric Colorimeter was used in this determination and in determinations of optical density of cell suspensions throughout this study. For nitrogen determinations a 470 millimicron filter was used and for determinations of culture turbidity a 590 millimicron filter was employed. In both the standard and the sample the amount of nitrogen was directly proportional to the optical density. The ratio of the optical density of the sample to that of the standard was multiplied by the amount of nitrogen in the standard and by the dilution factor to obtain the amount of nitrogen in one milliliter of the culture.

For the determination of ammonium nitrogen the cells were removed from the culture medium by centrifugation in a Servall Angle Centrifuge (Model 1). The medium was then made alkaline with sodium hydroxide and the liberated ammonia was distilled into four per cent boric acid. The method of dilution, treatment with Nessler's reagent, and calculation of nitrogen was the same as used for Kjeldahl nitrogen determinations.

### Growth

In experiments for the determination of the quantity of nitrogen fixed under anaerobic and aerobic conditions cultures were grown in 50 milliliters of the described medium contained in 250 milliliter Erlenmeyer flasks. The medium in each flask was inoculated with five milliliters of a suspension of strain N4. Samples were drawn from three flasks for initial nitrogen determinations. Three flasks were closed with cotton plugs and three were closed with rubber stoppers to prevent evaporation. Each of the remaining flasks was closed with a

rubber stopper fitted with a glass tube vent on which was placed a short length of rubber tubing closed with a screw type pinch clamp. Anaerobic conditions were provided by attaching the flasks to a gassing manifold connected to a mercury manometer and evacuating with a Duo Seal Vacuum Pump to about 0.15 atmospheres of pressure after which the flasks were refilled with high purity Linde tank nitrogen. Evacuation and subsequent filling with nitrogen were repeated four times to flush out any oxygen which might have remained as a result of incomplete evacuation. Both anaerobic and aerobic cultures were incubated on a New Brunswick rotary shaker (Model VS) at room temperature (approximately 27 degrees centigrade) for four days before final nitrogen determinations were made.

For growth curve experiments cultures were grown at room temperature in one liter of the described medium contained in a two liter Erlenmeyer flask equipped with a three hole rubber stopper which held an inlet sintered glass gas dispersion tube, a tube for sampling the culture, and a gas outlet tube. The inoculum was grown in 50 milliliters of the same medium under nitrogen gas for approximately 48 hours on the rotary shaker. The entire 50 milliliters of inoculum was used to inoculate one liter of medium. Nitrogen gas was continuously bubbled through the medium after being filtered through sterile cotton. Samples for nitrogen and turbidity determinations were taken at regular intervals by forcing a sample through the sampling tube with pressure obtained by closing the gas outlet tube.

The method employed in determining the effect of various partial pressures of nitrogen upon the rate of growth was essentially that used

by Pengra and Wilson (11) for hydrogen inhibition studies of A. aerogenes. A one liter culture was grown in the manner described above for growth curve experiments until it was in the early exponential phase of growth. It was then portioned out into 250 milliliter Erlenmeyer flasks which had fused to them Pyrex test tubes that fit the receptacle of the colorimeter. A sample was removed from each flask for determination of initial nitrogen. The flasks were closed with vented stoppers and anaerobic conditions were provided as previously described. After the fourth evacuation the desired experimental atmospheres were placed in the flasks. Helium was used to bring the total pressure in each flask to one atmosphere. The flasks were incubated at room temperature on the rotary shaker for a period of six hours. Growth was followed by tipping a sample of the contents of the flask into the sidearm tube and determining the optical density at one hour intervals. After incubation samples were removed for final nitrogen determinations.

Hydrogen inhibition

For hydrogen inhibition studies the method employed was identical to that described above for the determination of growth rates under various partial pressures of nitrogen except that in some experiments a longer incubation period was used.

For hydrogen inhibition studies of ammonium utilization a parent culture was grown in 200 milliliters of the described medium containing 150 micrograms of ammonium nitrogen per milliliter present as ammonium dihydrogen phosphate. This culture was grown under helium in a 500 milliliter Erlenmeyer suction flask until it was in the exponential



phase of growth. The culture was then portioned out into sidearm flasks. Experimental atmospheres were placed in them after flushing three times with helium. The cultures were incubated at room temperature on the rotary shaker. Growth was followed turbidimetrically.

### Hydrogenase

Dehydrogenase and some hydrogenase assays were conducted using Thunberg tubes which could be used in the colorimeter. Each tube contained four milliliters of M/15 phosphate buffer (pH 7.1) and one milliliter of  $10^{-4}$  molar methylene blue. To demonstrate glucose dehydrogenase activity some of the tubes received two milliliters of a one per cent solution of glucose. In each cap compartment was placed 0.5 milliliters of a washed whole cell suspension of strain N<sub>4</sub> containing about 150 micrograms of nitrogen per milliliter. Washed whole cell suspensions used throughout this study were prepared by centrifuging cells from an actively fixing culture, washing twice with 10 milliliters of M/15 phosphate buffer of the desired pH, estimating the nitrogen present, and resuspending in a quantity of buffer to provide the approximate amount of cell nitrogen per milliliter desired. Distilled water was added to bring the total volume in each tube to 7.5 milliliters. The desired atmospheres were placed in the tubes by evacuating them with an aspirator and refilling with the appropriate gas. Hydrogen was used for hydrogenase assays and helium was used in determinations for glucose dehydrogenase and endogenous activities. After allowing the tubes and contents to come to room temperature the content of each cap was emptied into the main tube and optical density

readings were made periodically.

The manometric techniques employed were in general those described by Umbreit, et al. (15). The Warburg apparatus used for these experiments was manufactured by Gilson Medical Electronics. The center wells of the Warburg flasks contained 0.2 milliliters of 20 per cent potassium hydroxide and a fluted filter paper for the absorption of carbon dioxide unless otherwise indicated. The total volume in each flask was adjusted to 3.2 milliliters with distilled water. All experiments were carried out with the water bath maintained at 32.5 degrees centigrade. In all experiments the temperature of the flask was allowed to equilibrate in the water bath for ten minutes prior to tipping the contents of the sidearms into the main compartments. Readings were made at the time of tipping and every five minutes thereafter for a period of 30 minutes. In all manometric assays a control organism which was known to possess hydrogenase was used parallel to strain N4. The organisms which were used for this purpose were A. aerogenes strain M5al and Azotobacter vinelandii strain 0 (Azotobacter agilis). Whole cell suspensions and cell-free extracts of Achromobacter N4 contained substantially greater quantities of nitrogen per milliliter than did similar preparations of the control organism. The purpose of this was to increase the possibility of detecting a small quantity of activity.

For the formic hydrogenlyase assay the main compartment of the Warburg flask received 1.5 milliliters of M/15 phosphate buffer (pH 6.7) and one milliliter of 0.25 molar sodium formate. Only one half of the flasks received potassium hydroxide and a fluted filter paper for the

absorption of carbon dioxide. Any pressure increase in these flasks would indicate the production of hydrogen. In the flasks without the carbon dioxide absorbant the pressure change would be from both hydrogen and carbon dioxide. The sidearms received 0.2 milliliters of a washed whole cell suspension of Achromobacter N4. A control set of flasks was set up using 0.2 milliliters of a washed whole cell suspension of A. aerogenes strain M5a1 containing about 110 micrograms of nitrogen. The flasks were fitted to the Warburg manometers and flushed with flowing helium for several minutes using a gassing manifold.

Manometric assays for hydrogen uptake using whole cell suspensions were set up as follows: The main compartment of the Warburg flask received 1.5 milliliters of M/15 phosphate buffer and the desired amount of hydrogen acceptor which was 25 micromoles of methylene blue, 40 micromoles of methyl viologen, 40 micromoles of benzyl viologen, 20 micromoles of Janus green or 125 micromoles of potassium ferricyanide. Most experiments were carried out at a pH of 8.0, which was optimum for the control organism. The suspension of the control organism contained about 100 to 250 micrograms of nitrogen per milliliter. In most experiments 0.2 milliliters of cell suspension were added to the sidearm of each flask. With the aid of a gassing manifold the flasks were flushed with hydrogen for 10 minutes.

Assays for hydrogen uptake using cell-free extracts were conducted in the same manner as were assays using washed whole cell suspensions. In these experiments, however, the pH was 7.1 and the quantity of nitrogen added per flask as cell-free extract was increased

about 20 times. For the preparation of cell-free extracts relatively large quantities of cells were needed. Therefore, the cells were grown in two liter culture flasks as described on page 8. The cells were harvested by centrifugation and were then stored under hydrogen at -5 degrees centigrade or were used immediately for preparation of cell-free extracts. Cell-free extracts were prepared by actively grinding a small quantity of cells with an equal quantity of levigated alumina for 10 minutes with a chilled mortar and pestle. Several milliliters of M/15 phosphate buffer having a pH of 7.1 was used for extraction. The extraction medium was then centrifuged for five minutes in the Sorvall Angle Centrifuge at a speed sufficient to provide a force of 2500 times gravity at the tip of the tube. The clear supernatant fluid was used as the cell-free extract.

Attempts to demonstrate the Knallgas reaction in this organism were carried out by placing 1.5 milliliters of M/15 phosphate buffer having a pH of 8.0 in the main compartment and 0.2 milliliters of a washed whole cell suspension in the sidearm. *A. vinelandii* strain 0 was used as the positive control. The cell suspensions contained about 250 to 300 micrograms of nitrogen per milliliter. After attaching the flasks to the Warburg manometers a gas mixture consisting of 50 per cent hydrogen and 50 per cent oxygen by volume in a one gallon reservoir was displaced with water and flushed through one half of the flasks. The remaining flasks containing air served as controls for endogenous respiration.

The assay for the evolution of hydrogen from reduced methyl viologen was essentially that of Peck and Gest (9). In this assay

the main compartment of the Warburg flasks contained 1.5 milliliters of M/15 phosphate buffer (pH 7.6), 0.1 milliliter of the cell suspension containing 40 to 60 micrograms of nitrogen, and 0.1 milliliter of cysteine hydrochloride solution. This solution was made just prior to use by dissolving 50 milligrams of cysteine hydrochloride in five milliliters of M/15 phosphate buffer having a pH of 7.6. The final pH of the cysteine solution was adjusted to 7.6 with 1.0 normal sodium hydroxide. The sidearms contained 32 micromoles of methyl viologen in 0.4 milliliters of water. The methyl viologen contained in the sidearms was reduced during the gassing operation by the addition of 0.2 milliliters of a sodium hydrosulphite solution. This solution was prepared by dissolving 210 milligrams of sodium hydrosulphite in three milliliters of water and adjusting the pH to 7.6 with 0.125 normal sodium hydroxide. Helium was used to displace the air from the flasks.

## RESULTS AND DISCUSSION

Growth

An experiment was carried out to determine the quantity of nitrogen fixed by Achromobacter N4 when growing on the described medium under anaerobic and aerobic conditions. The results of this experiment are given in Table I.

TABLE I. INFLUENCE OF AERATION ON FIXATION

		Nitrogen content ugm./ml.	Nitrogen fixed ugm./ml.
Anaerobic	Control	10	—
	Culture 1	85	75
	Culture 2	75	65
	Culture 3	76	66
Aerobic*	Control	10	—
	Culture 1	9	0
	Culture 2	8	0
	Culture 3	7	0
Aerobic**	Control	10	—
	Culture 1	10	0
	Culture 2	7	0
	Culture 3	9	0

\*Flasks closed with cotton plugs.

\*\*Flasks closed with rubber stoppers to prevent evaporation.

The depressing effect of oxygen was found to be greater in this experiment than in studies by Jensen (7) and Proctor and Wilson (14) in that no fixation at all was obtained under aerobic conditions.

When studying the physiology of nitrogen fixation it is often necessary to conduct experiments during a phase of growth when the

organisms are actively fixing nitrogen. For this reason a study was made of the growth curve of Achromobacter. Figure 1 is a typical growth curve of strain N4 when grown under nitrogen gas in the presence of about 30 micrograms of ammonium nitrogen per milliliter of medium. The second lag phase was also observed in studies by Proctor and Wilson (14) and is similar to that demonstrated by Pengra and Wilson (11) for A. aerogenes. Figure 2 illustrates the correlation between growth and the disappearance of ammonium nitrogen. The fact that the total nitrogen does not increase until the end of the second lag phase (Figure 1) indicates that fixation of atmospheric nitrogen does not occur until the end of the second lag phase. The fact that combined nitrogen nears depletion at the same time that the initial growth phase reaches a maximum (Figure 2) indicates that the initial growth phase is a result of growth on ammonium nitrogen. These facts indicate that strain N4 will utilize ammonium nitrogen in preference to atmospheric nitrogen and suggest that the second lag phase may be a period of induction of the nitrogen fixing system.

The effect of various partial pressures of nitrogen upon the rate of fixation is illustrated in Figure 3. In this experiment optical density closely paralleled cell nitrogen. This close relationship indicated that optical density could be used as a measure of nitrogen fixed in some experiments.

#### Hydrogen inhibition

Hydrogen has been shown to inhibit nitrogen fixation by most of the nitrogen fixing agents thus far reported. Figure 4 illustrates the

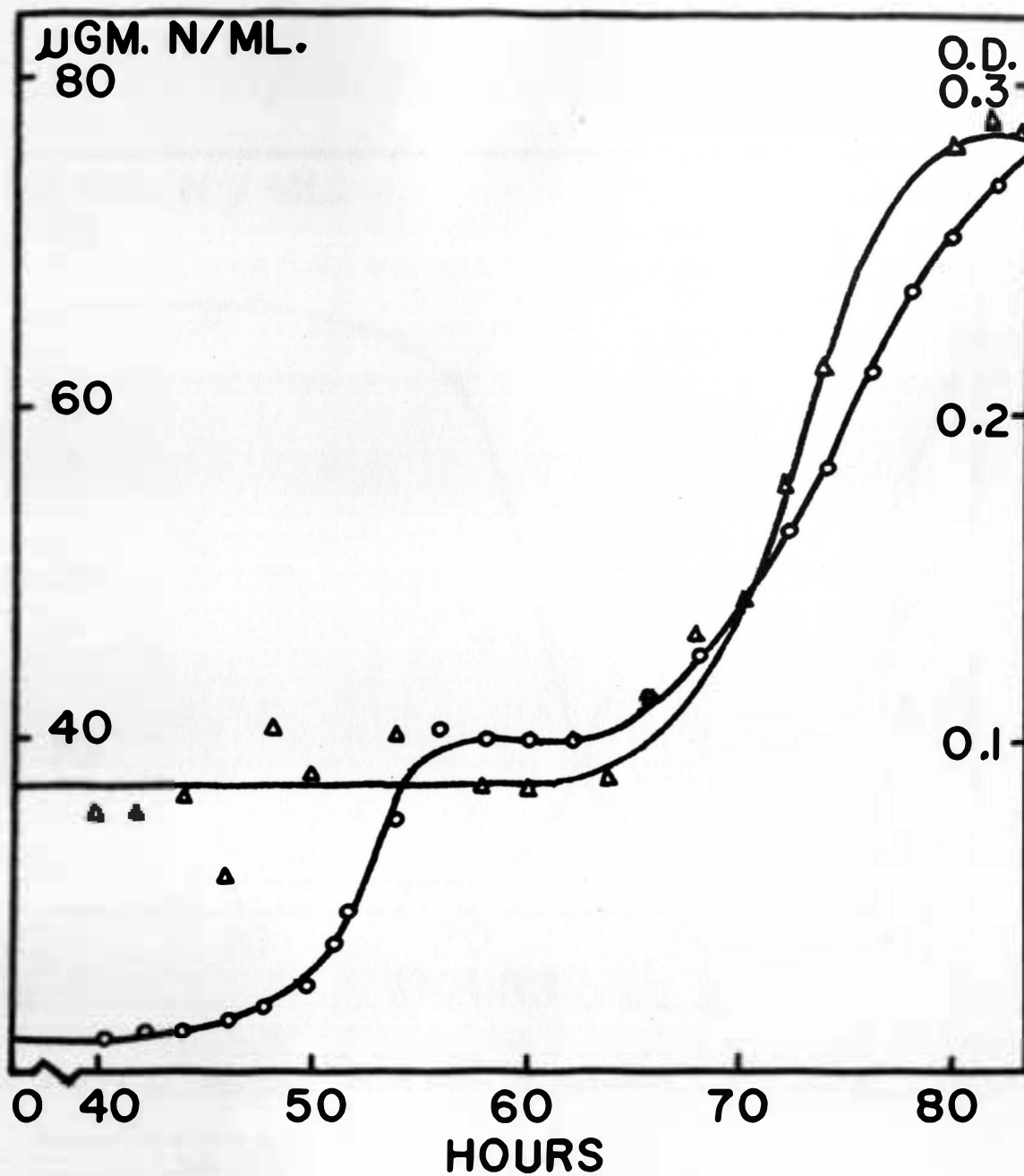


Figure 1. Growth Curve of *Achromobacter N4* When Grown in the Presence of a Limited Amount of Ammonium Nitrogen

Culture turbidity     $\circ - \circ$   
 Total nitrogen       $\Delta - \Delta$



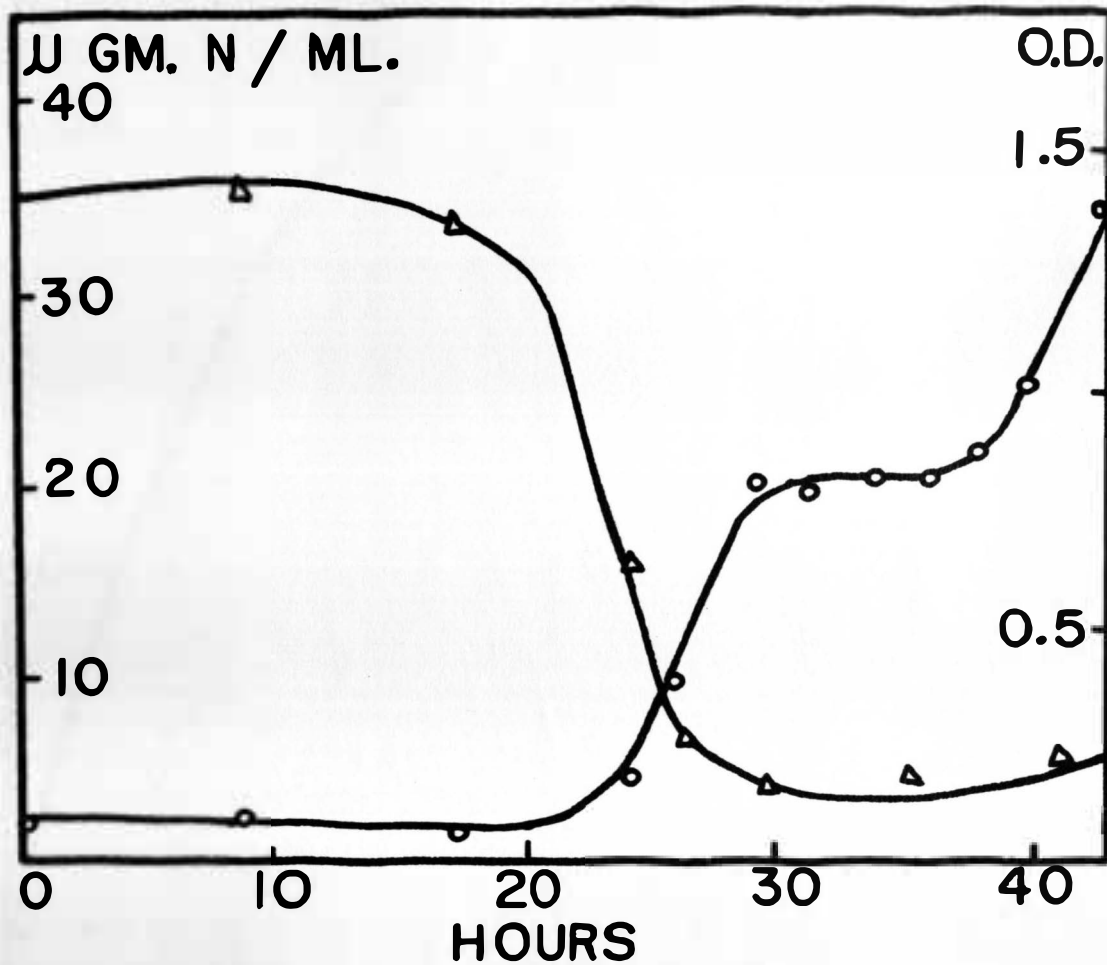


Figure 2. Utilization of Ammonium Nitrogen by Achromobacter N4

Ammonium nitrogen  $\triangle$ — $\triangle$   
 Culture turbidity  $\circ$ — $\circ$

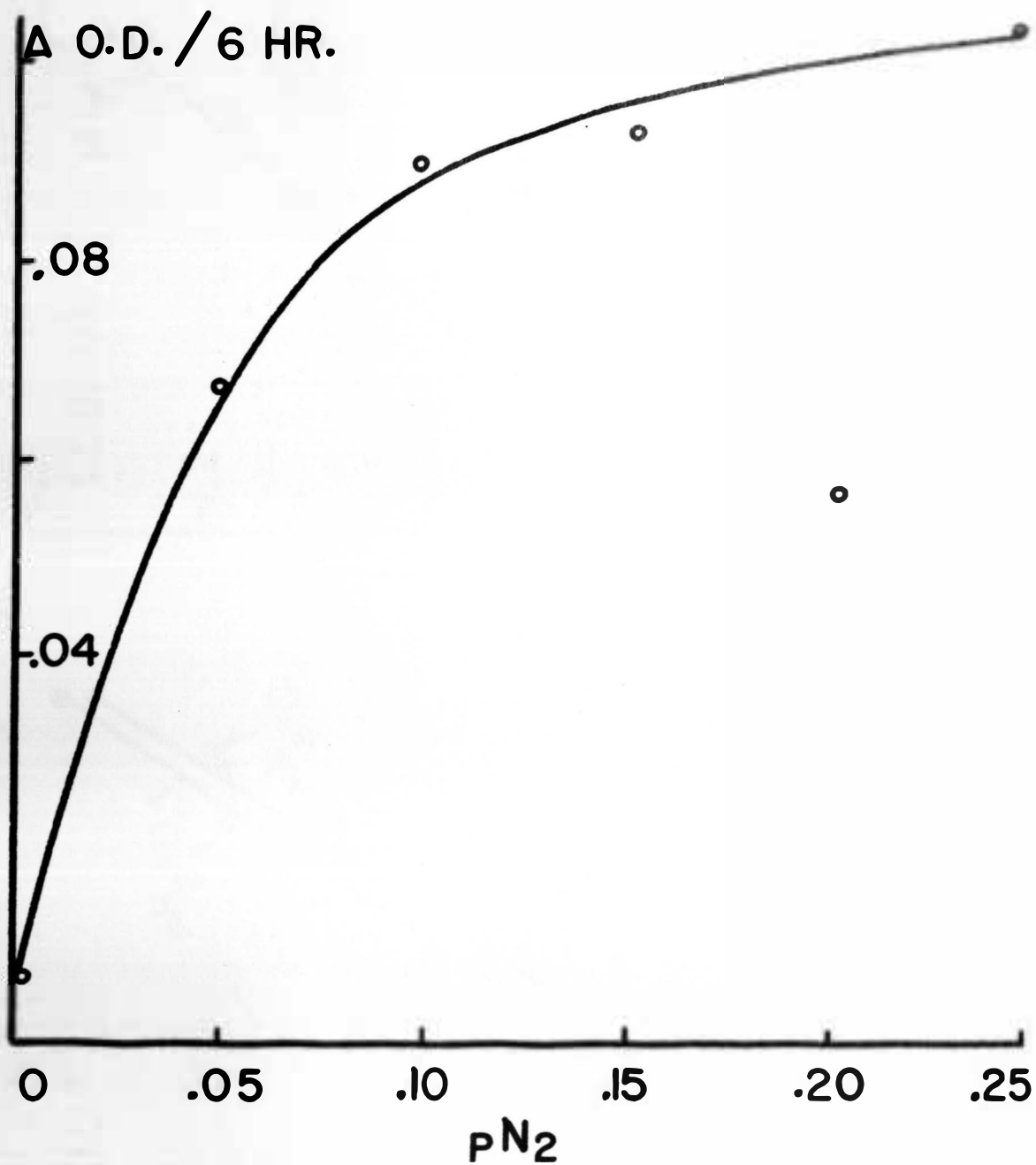


Figure 3. The Effect of Various Partial Pressures of Nitrogen Upon the Rate of Fixation by Achromobacter N<sub>4</sub>

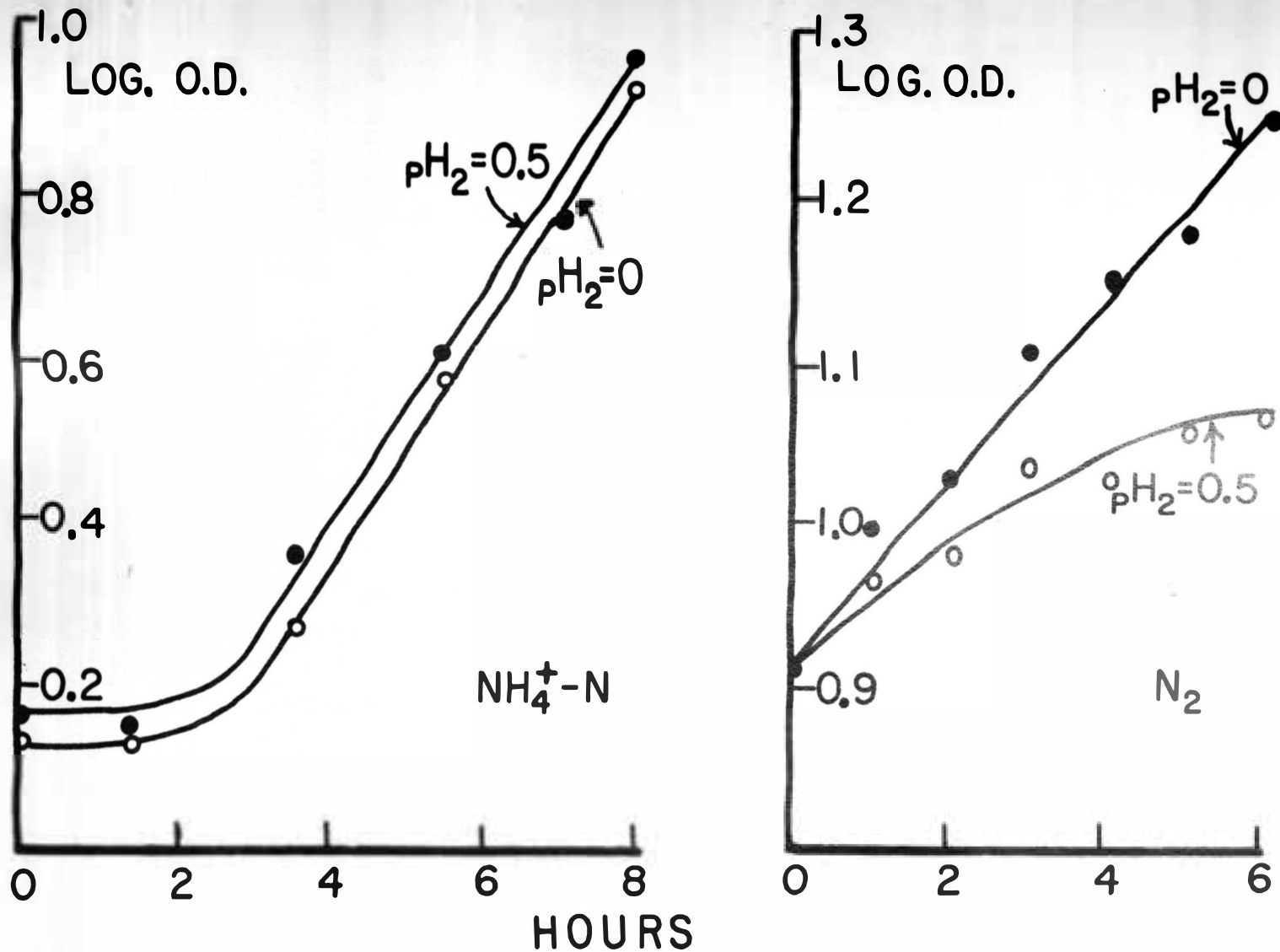


Figure 4. The Effect of Hydrogen Upon the Assimilation of Gaseous and Ammonium Nitrogen by *Achromobacter N4*.

specific inhibitory effect of hydrogen on nitrogen fixation by Achromobacter N4. Table II shows the inhibitory effect of several partial pressures of hydrogen on nitrogen fixation by this organism.

TABLE II. INHIBITION OF NITROGEN FIXATION BY HYDROGEN

<u>% H<sub>2</sub> in atmosphere*</u>	<u>Initial N ugm./ml.</u>	<u>Final N ugm./ml.</u>	<u>N fixed in 7 hr. ugm./ml.</u>	<u>% inhibition</u>
0	31	50	19	—
25	28	39	11	42
50	32	38	6	68

\*The atmosphere above all cultures had a p<sub>H<sub>2</sub></sub> of 0.25. Helium was used as the inert gas to bring the total pressure in each flask to one atmosphere.

In hydrogen inhibition experiments where turbidity of the cultures was used as a measure of growth it was noticed that growth was not exponential. Therefore, the measure of nitrogen fixed during these experiments could not be used in calculation of growth rate constants. Because of this it could not be determined whether the inhibition was competitive or non-competitive.

### Hydrogenase

All of the nitrogen fixing agents thus far investigated are known to possess hydrogenase. The fact that nitrogen fixation by strain N4 is inhibited by hydrogen indicates that this organism must possess some enzyme such as hydrogenase which activates molecular hydrogen. Thus far no one has been able to demonstrate hydrogenase in this

organism using conventional manometric and Thunberg hydrogenase assays.

Typical results of Thunberg hydrogenase and glucose dehydrogenase assays which were made during this study using washed whole cell suspensions of strain M4 are shown in Figure 5. In experiments where cells were not washed prior to use it was found that a considerable amount of endogenous dehydrogenase activity was present.

The formic hydrogenlyase system is by definition an enzyme system which brings about the cleavage of formate to form hydrogen gas and carbon dioxide. There has been some controversy as to whether formic hydrogenlyase is a distinct enzyme, or whether its action is a result of the coupled effects of formic dehydrogenase and hydrogenase. The fermentation of sugar by strain M4 produces no gas. Although this gasless fermentation implies that the organism has no hydrogenlyase system it was felt that an attempt to demonstrate such a system was warranted because of the possible participation of hydrogenase in this system. Figure 6 gives the results of a typical experiment showing that both carbon dioxide and hydrogen were formed from formate by the control organism but not by the Achromobacter strain.

It is realized that in hydrogenase assays involving the uptake of hydrogen the ideal hydrogen acceptor would be the physiological one functioning in the cell. However, the role of hydrogenase in nitrogen fixation is not definitely established and therefore it was necessary to select several artificial acceptors for use in these assays. The qualitative results of manometric assays for hydrogen uptake using washed whole cell suspensions are given in Table III.

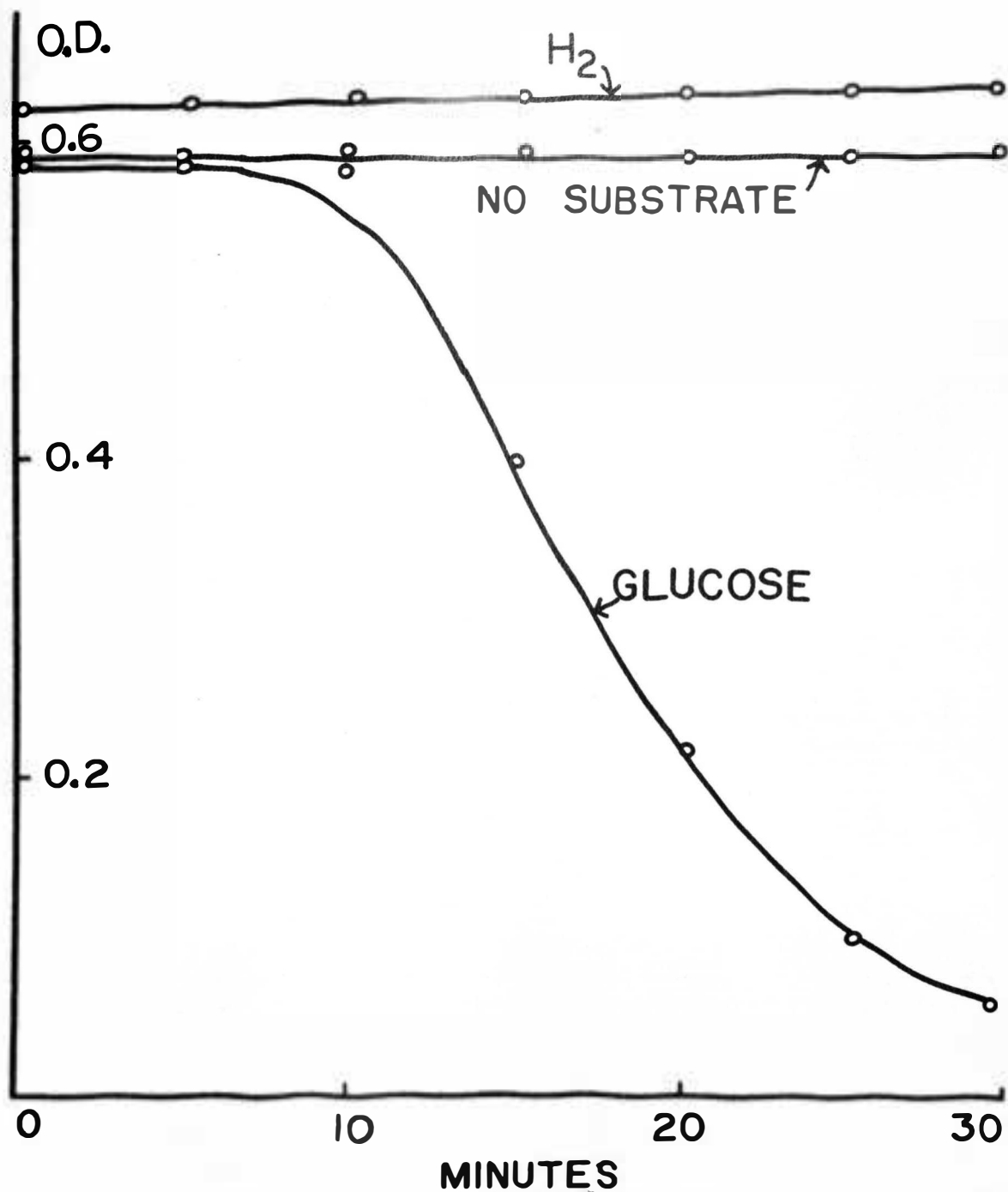


Figure 5. Methylene Blue Reduction by Intact Cells of Achromobacter N4 Using Hydrogen and Glucose.

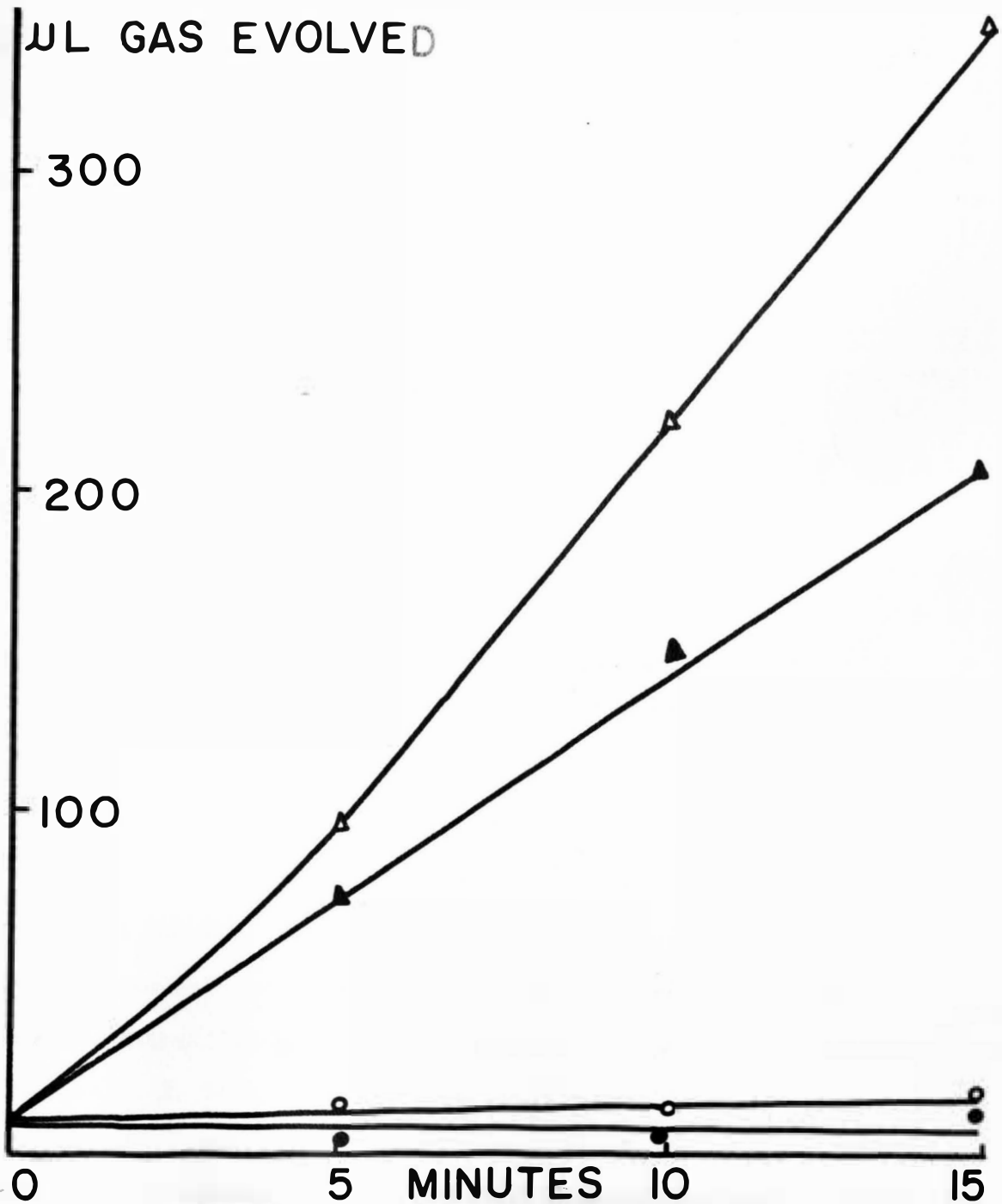


Figure 6. Formic Hydrogenlyase Activity Using Washed Whole Cell Suspensions

*Aerobacter aerogenes* strain M5a1 with KOH ▲—▲ without KOH △—△  
*Achromobacter N4* with KOH ●—● without KOH ○—○

TABLE III. HYDROGENASE ACTIVITY OF WASHED WHOLE CELL SUSPENSIONS  
WHEN ASSAYS FOR HYDROGEN UPTAKE WERE USED

Hydrogen acceptor	Control organism used	Hydrogenase activity of control organism	Hydrogenase activity of strain H4
Methylene blue	AVO*	+	-
	M5al**	+	-
Methyl viologen	AVO	-	-
	M5al	-	-
Benzyl viologen	M5al	-	-
Janus green	AVO	+	-
Potassium ferricyanide	AVO	+	-

\*Azotobacter vinelandii strain 0 (Azotobacter agilis)

\*\*Aerobacter aerogenes strain M5al

From the results obtained with whole cell suspensions of organisms known to possess hydrogenase (Table III) it appears that certain hydrogen acceptors such as methyl viologen and benzyl viologen are not satisfactory for use with whole cell suspensions. The fact that Pengra (10) used these acceptors in demonstrating hydrogenase activity in cell-free extracts of A. aerogenes suggested that impermeability might be involved. In view of this several hydrogenase assays were conducted using cell-free extracts in which impermeability would not be a problem. Results of one such assay in which benzyl viologen was used as the hydrogen acceptor are given in Figure 7. Figure 8 illustrates the results obtained when methyl viologen and methylene blue were employed as the hydrogen acceptors.



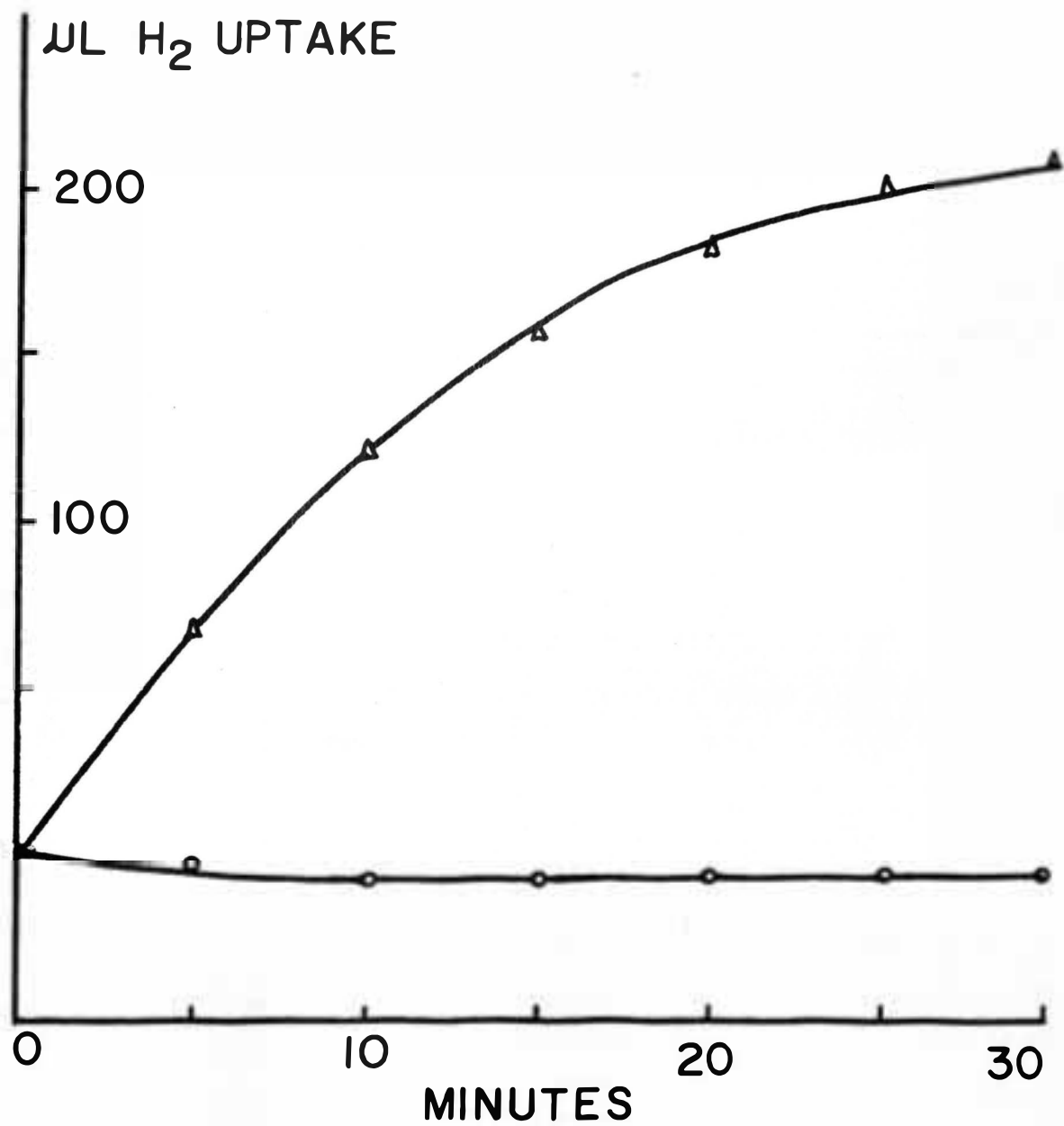


Figure 7. Hydrogenase Activity of Cell-free Extracts Using Benzyl Viologen as the Hydrogen Acceptor

*Aerobacter aerogenes* strain M5a1  $\Delta-\Delta$   
*Achromobacter* N4  $\circ-\circ$

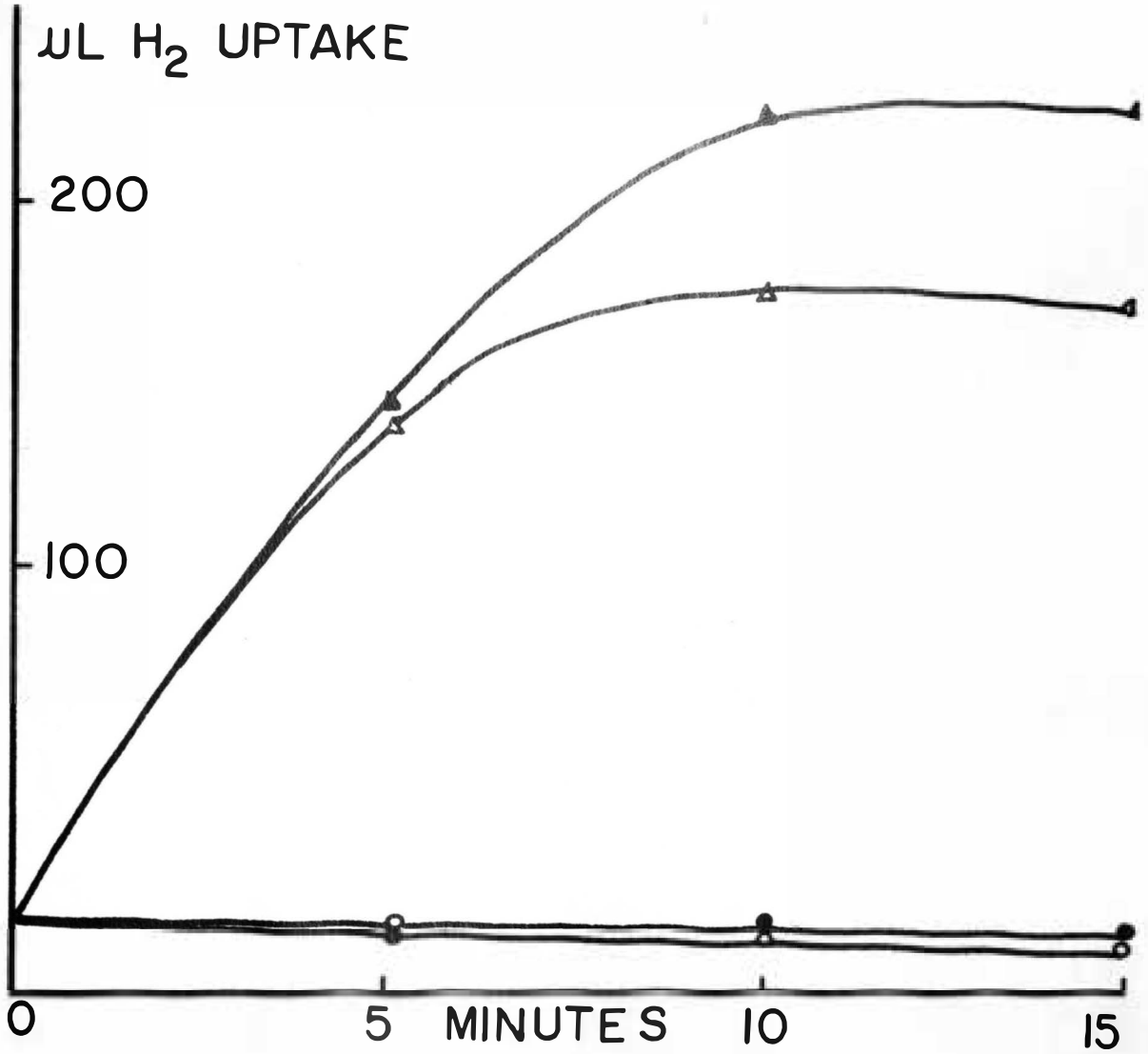


Figure 8. Hydrogenase Activity of Cell-free Extracts Using Methyl Viologen and Methylene Blue as the Hydrogen Acceptors

- Aerobacter aerogenes* strain M5a1 - methylene blue ▲—▲
- Aerobacter aerogenes* strain M5a1 - methyl viologen △—△
- Achromobacter N4* - methylene blue ●—●
- Achromobacter N4* - methyl viologen ○—○

An enzyme such as hydrogenase is considered essential for the oxidation of molecular hydrogen. In view of this an attempt was made to demonstrate the Knallgas reaction where molecular hydrogen is oxidized to form water. The results of one such experiment are shown in Figure 9.

Since hydrogen is more often evolved than oxidized or fixed by bacteria under laboratory conditions the assay of Peck and Gest (9) was employed whereby hydrogen is evolved from reduced methyl viologen. The results of this experiment are given in Figure 10.

From the foregoing description of results it is evident that conventional manometric and Thunberg methods of assay fail to demonstrate the presence of hydrogenase in Achromobacter N4. The results of experiments conducted by Proctor and Wilson (14) in which the deuterium-hydrogen exchange reaction was used as the method of assay indicate that hydrogenase is present. Confirmatory experiments should be conducted to establish definitely the presence of hydrogenase in Achromobacter N4.

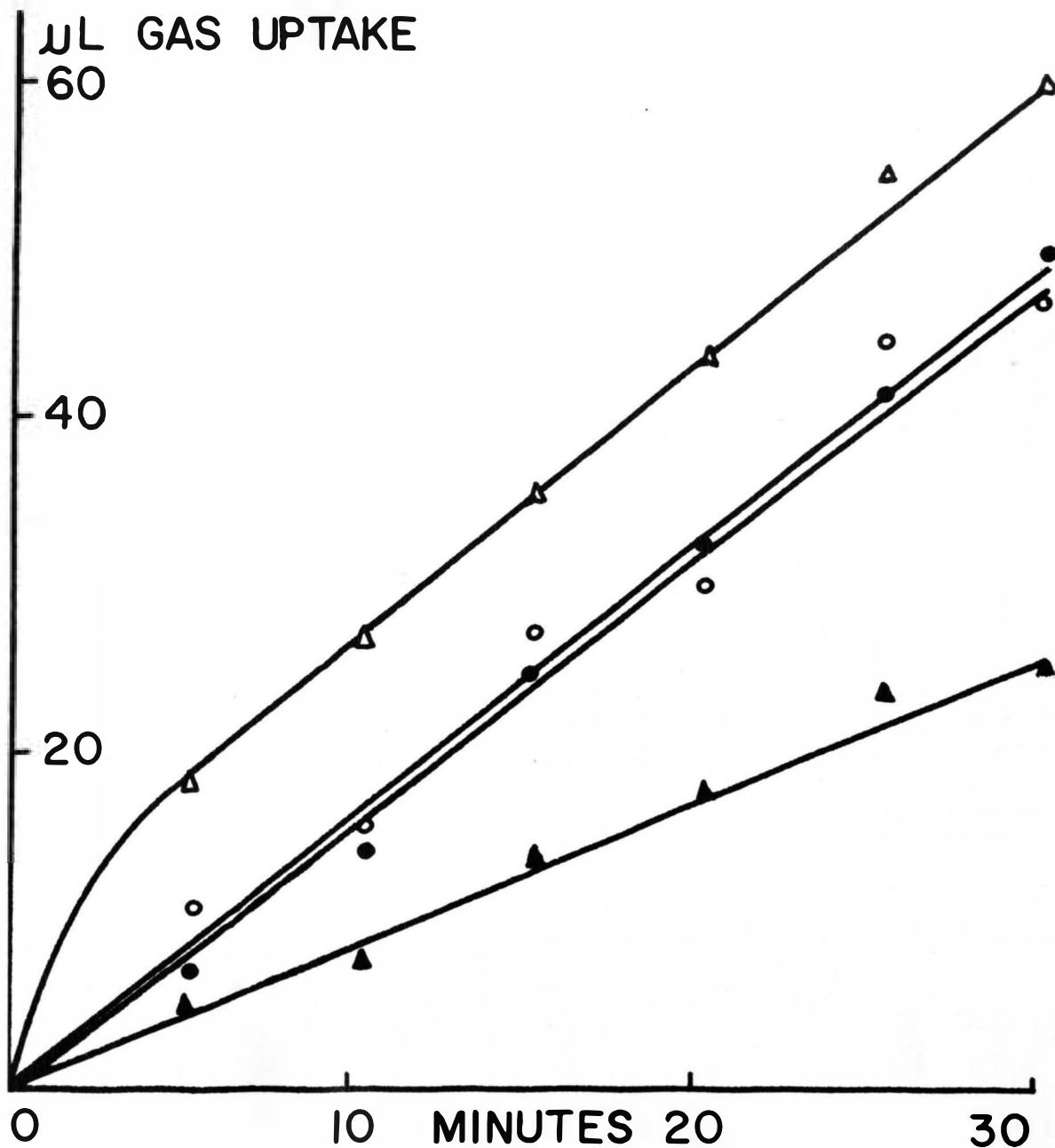


Figure 9. Oxidation of Molecular Hydrogen by Oxygen

*Azotobacter vinelandii* strain 0 - hydrogen and oxygen  $\triangle$ — $\triangle$   
*Azotobacter vinelandii* strain 0 - endogenous control  $\blacktriangle$ — $\blacktriangle$   
*Achromobacter* N4 - hydrogen and oxygen  $\circ$ — $\circ$   
*Achromobacter* N4 - endogenous control  $\bullet$ — $\bullet$

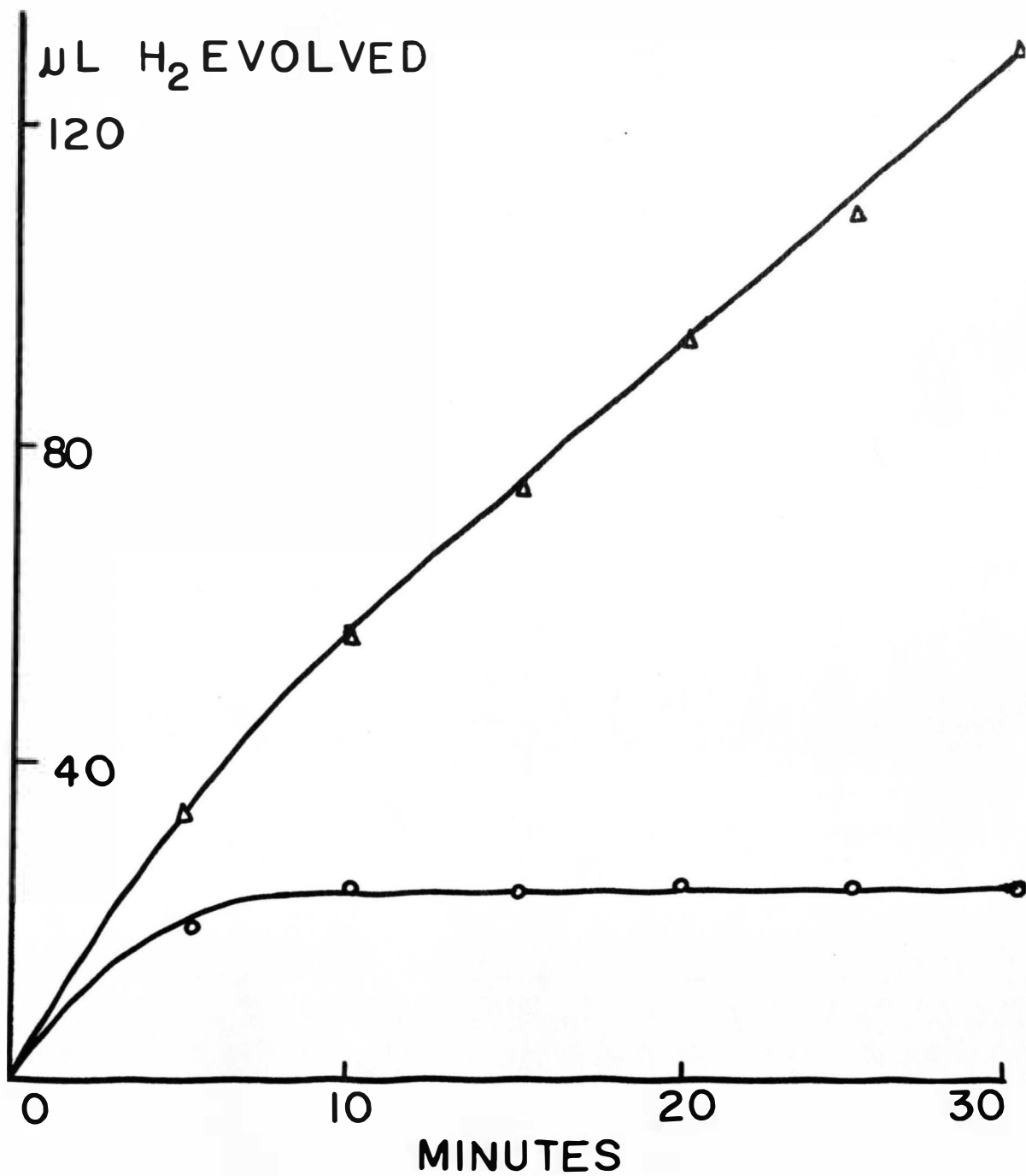


Figure 10. Evolution of Hydrogen from Reduced Methyl Viologen

Aerobacter aerogenes strain M5a1  $\triangle$ — $\triangle$   
Achromobacter N4  $\circ$ — $\circ$

### SUMMARY

The physiology of nitrogen fixation by a nitrogen fixing species of the genus *Achromobacter* was studied. It was found that this organism produces relatively large amounts of acid during its gasless fermentation of carbohydrate. When growing anaerobically in a medium of a high buffering capacity it was found to fix about 70 micrograms of nitrogen per milliliter of medium. Fixation under aerobic conditions was not obtained. Growth curve studies revealed that it will utilize ammonium nitrogen in preference to gaseous nitrogen and suggested that at least one of the enzymes necessary for nitrogen fixation in this organism is inducible. Hydrogen was shown to be a specific inhibitor of the nitrogen fixing system. A hydrogenlyase system could not be demonstrated. Using conventional Thunberg and manometric methods of assay no hydrogenase activity was found in either whole cell suspensions or cell-free extracts of this organism.

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