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HYDROGEN PRODUCTION FROM THE PHOTOSYNTHETIC BACTERIUM, RHODOPSEUDOMONAS SPHAEROIDES

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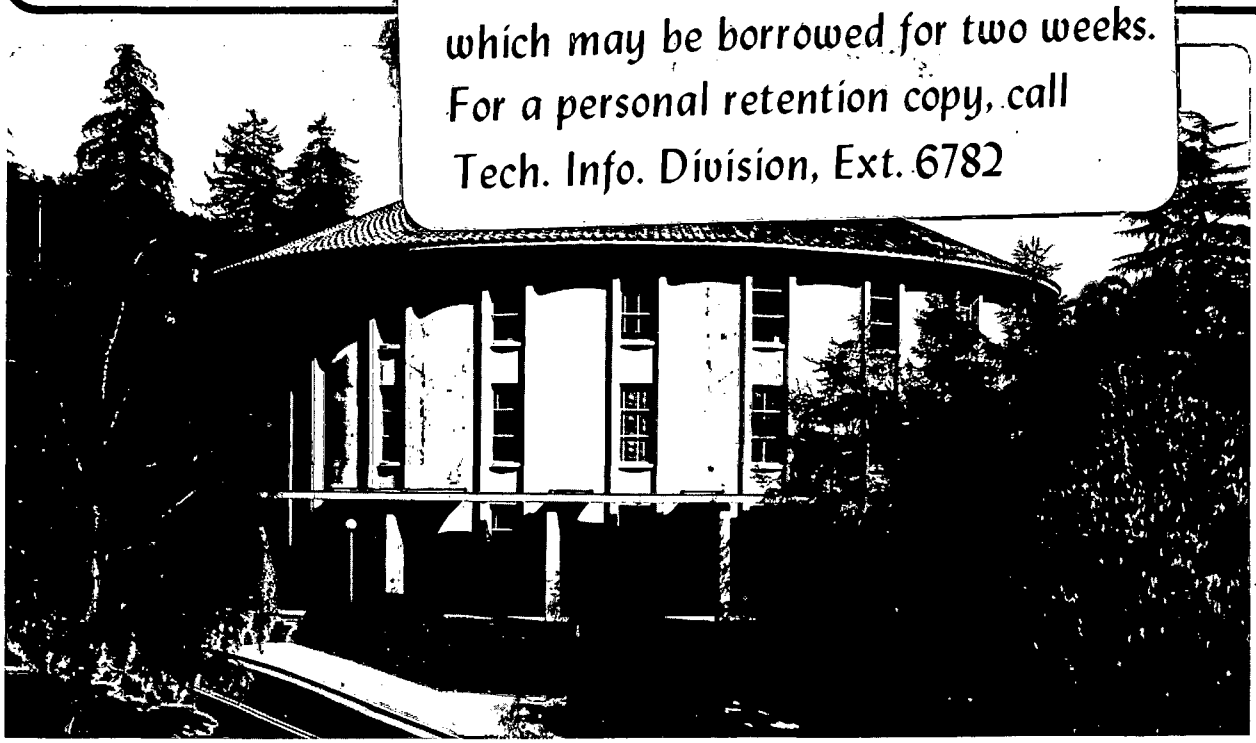

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HYDROGEN PRODUCTION FROM THE PHOTOSYNTHETIC BACTERIUM, *RHODOPSEUDOMONAS SPHAEROIDES*

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by

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ABSTRACT

The photosynthetic bacterium, *Rhodopseudomonas sphaeroides* can produce H_2 gas under certain conditions when supplied with an appropriate reduced carbon source. It was determined that the enzyme nitrogenase is responsible for this activity. It was observed that maximum rates of H_2 biosynthesis were achieved when the organism was grown anaerobically in the light in a medium containing minimal amounts of reduced nitrogen. An atmosphere low in N_2 also increased H_2 yields.

The organism can utilize lactate, malate, fructose or sucrose to produce H_2. Glucose and gluconate are not normally so utilized. Growth on glucose yields gluconate and a decrease in culture pH. Mutant strains were isolated that could utilize gluconate or glucose to produce H_2 and did not lead to a decrease in culture pH. The glucose-utilizing strain (*Glc*$^+$) was highly efficient, capable of essentially complete conversion of glucose to H_2 and CO_2.

The energy charge, as described by Atkinson, was about 0.5 regardless as to whether the organism was producing H_2. Total nicotinamide adenine dinucleotide levels were three-times that of nicotinamide adenine dinucleotide phosphate. The steady-state ratios of reduced to oxidized forms of both of these compounds was about 0.2.

Ammonium ion completely inhibited H_2 production at 1 mM. O_2 inhibited H_2 production completely and irreverisbly in vivo when present above 8% in the atmosphere. N_2 did not completely suppress H_2 production at any level. Carbon monoxide at 10% in the atmosphere did not affect metabolism or inhibit H_2 production. Monofluoroacetate inhibited H_2 production from 10-70% depending on the strain and the carbon compound used for substrate.

For cells grown on malate, fructose, or glucose, storage material formed was almost entirely polyglucose. During H_2 production, amino acids were not produced, even when an amino group donor such as glutamate was present.

Metabolic pathways in glucose utilization were strain dependent. Wild-type and Glc^+ mutant cells metabolized this compound primarily by the Embden-Meyerhof pathway. The tricarboxylic acid cycle appeared to be operative under all conditions. The Glc^+ strain had an enhanced ability to utilize glucose without the generation of gluconate, and the glycolytic pathway and the tricarboxylic acid cycle were more active.

CO_2 fixation in cultures producing H_2 photoheterotrophically is minimal and occurs primarily via phosphoenolpyruvate carboxylase.

Kenneth Sauer

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HYDROGEN PRODUCTION FROM THE PHOTOSYNTHETIC BACTERIUM, *RHODOPSEUDOMONAS SPHAEROIDES*

Bruce Alan Macler

In partial fulfilment of the degree of Doctor of Philosophy

University of California, Berkeley

1980

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INTRODUCTION

It was many years ago that Gest and Kamen observed that *Rhodospirillum rubrum*, a purple non-sulfur bacterium, released H_2 gas when grown anaerobically in the light (28,29). Since then, their work has been expanded by themselves and others to uncover the physiological requirements and biochemical systems of H_2 biosynthesis in the photosynthetic bacteria and in other organisms. The enzymes involved in H_2 production, nitrogenase and hydrogenase, have been isolated and characterized for a variety of organisms (13,64). Factors involved in the regulation of these systems, including the control of synthesis and function of the enzymes, the generation of energy and reducing power and the metabolic state of the cell, have been explored and described (9,74,82,88).

In reviewing the literature at length, it became clear to Dr. Al Bassham, Dr. Dick Pelroy and me that *R. rubrum* and the similar *Rhodopseudomonas sphaeroides* would work very well in a solar energy-waste conversion system. It would be useful to the world to have a simple, high yield system for the generation of fuel from readily available materials. A system using sunlight for energy and waste products for raw materials to produce hydrogen gas was exciting to us and we had visions of large ponds of paper-mill waste or sewage, covered with transparent collecting canopies, full of actively growing bacteria bubbling off H_2 gas to run automobiles or heat homes. This seemed a practical idea, provided that the organism was up to the task. It was clear that no one had looked carefully at bacterial H_2 production with an eye towards

understanding H_2 production *in vivo*. Most of the literature dealt with isolated enzymes or broken-cell preparations. My interest became one of looking at the organisms *in vivo* with the aim of understanding them well enough to be able to optimize the conditions of H_2 production.

My initial work dealt with the growth conditions and physiology of the H_2 production phenomenon. I looked at the effects of media and carbon sources, pH, light, atmosphere and metabolic inhibitors on H_2 biosynthesis. *R. sphaeroides* became the organism of choice for its ease of handling, the wide variety of culture conditions under which it can grow and the large amounts of H_2 that it can produce. The ultimate product of this work was a 10 liter long-term culture of *R. sphaeroides* that could be maintained producing H_2 for weeks.

Growth of this organism on glucose was unpredictable. Generally H_2 was not produced. Occasionally, however, small amounts of H_2 were produced after several days of stressed conditions on minimally-nutritive medium. I began a project to isolate strains of *R. sphaeroides* that could use glucose to make H_2 in large amounts. Eventually, mutant strains for other substrates were isolated as well. These results lent credence to the idea of using organic waste material as growth substrates for the organism, since it seemed generally possible to find strains that produced H_2 when grown on specific materials.

At this point I became interested in radiotracer experiments to uncover the metabolic pathways actually occurring in *R. sphaeroides* for the specific substrates and conditions under which it was grown. The literature on the subject reported the demonstration *in vitro* of the

necessary enzymes for several degradative pathways, including the Embden-Meyerof pathway (2,3,59,80), the oxidative pentose phosphate pathway (2,31,32,52) and the Entner-Doudoroff pathway (17,76), but there have been no reports as to what occurs *in vivo*. Using the mutant and wild-type strains, I have been able to describe the flow of carbon from substrate to end products during H_2 synthesis.

R. sphaeroides is a member of the purple non-sulfur bacteria, which is one of the three families of photosynthetic bacteria. The photosynthetic bacteria are known to contain only one photosystem (74,88). This means that an external source of electrons must be provided for the generation of reducing power. There is no light-mediated photolysis of water as in the higher plants. Also, the primary light reaction in these organisms yields a product that is more electropositive than that seen in higher plants, meaning that the reduction of pyridine nucleotides or ferredoxins is not directly light mediated (64). The photosystem does generate ATP through cyclic phosphorylation (88).

The purple non-sulfur bacteria can grow in several different systems. Autotrophic growth with H_2, CO_2 and NH_4^+ anaerobically in the light has been demonstrated (3,23,26,64). Photoheterotrophy with organic electron donors such as alcohols, carboxylic and fatty acids and sugars, has been seen both aerobically and anaerobically (3,17,24,31,37,46,49, 59, 63,76). Aerobic and anaerobic growth on organic compounds in the dark has also been observed (54,55,70,80,86). The growth rates for dark anaerobic and light aerobic conditions are low, however (17,55,70,80).

Enzymatic studies have suggested that R. rubrum and R. capsulata fix CO_2 via either the reductive pentose phosphate (Calvin) cycle (2,3,26), or through a reductive tricarboxylic acid (TCA) cycle (24,26). This has been observed both photoautotrophically with H_2 as the electron source or photoheterotrophically on organic substrates. The enzymes necessary for oxidation of organic compounds to yield reducing power or ATP have been shown to occur both aerobically or anaerobically for the TCA cycle, glycolysis, the oxidative penthose phosphate cycle and the Entner-Doudoroff pathway (3,17,23,31,63,76).

Although low rates of H_2 biosynthesis occur for dark-grown cultures of these bacteria (70), the light-mediated process is quantitatively more significant and has therefore attracted more study. For those compounds studied, the process under certain conditions yields CO_2 and H_2 as the sole products of conversion (31). Equations 1 and 2 show the stoichiometry of this photoconversion process for glucose and malate:

$$C_6H_{12}O_6 + H_2O \xrightarrow{\text{LIGHT}} 12\ H_2 + 6\ CO_2 \qquad (1)$$

$$C_4H_6O_5 + H_2O \xrightarrow{\text{LIGHT}} 6\ H_2 + 4\ CO_2 \qquad (2)$$

Note that although water does not provide electrons by photolysis, water apears to provide some of the electrons for proton reduction through metabolism. Depending on the organic substrate, there may or may not be any net storage of chemical energy in H_2, but the energy to drive the conversion at a significant rate comes from light, presumably via energy stored in ATP formed by photophosphorylation, photoelectron transport or both. H_2 production has been demonstrated in the dark in the presence of ATP (Madigan, Wall and Gest, in press).

H_2 can be produced enzymatically by hydrogenase or, under certain conditions, by the nitrogen-fixing enzyme nitrogenase. Hydrogenase catalyzes the reaction of $2\ H^+ + 2\ e^- \rightleftharpoons H_2$. This reaction is ATP independent, although ATP might be required for the generation of reductants of sufficient electro-negativity to serve as hydrogenase substrates. Reduced ferredoxins are postulated to be the primary electron donor *in vivo*. A variety of organic and inorganic electro donors catalyze this reaction *in vitro*. Hydrogenases as a class are reversibly inhibited by O_2, which appears to compete with protons as an electron acceptor in the reduction process. Hydrogenases are non-competitively inhibited by carbon monoxide, which may affect the obligatory electron transport chain. Cytochrome C_3 is generally required for activity (85). The catalyzed reaction is reversible and may serve as an uptake mechanism to yield reducing power when *R. sphaeroides* is grown autotrophically on H_2.

Nitrogenase catalyzes the reduction of dinitrogen gas to ammonia: $N_2 + 6\ e^- + 6\ H^+ + 12\ ATP \rightarrow 2\ NH_3 + 12\ ADP + 12\ P_i$. The energy requirements for this reaction has not been precisely determined, but it is believed to be at least 12 moles of ATP per mole N_2 reduced (21,32). This energy is not conserved in the products. Nitrogenase also catalyzes the reduction of protons: $2\ H^+ + 2\ e^- + 4\ ATP \rightarrow H_2 + 4\ ADP + 4\ P_i$. Again, the absolute ATP requirement is not known. The two reactions appear to compete for cellular reducing power. H_2 production is highest at low partial pressures of N_2 and decreases as N_2 increases (28,29). Nitrogenases are irreversibly inhibited by O_2. They are non-competi-

tively inhibited by CO, which may affect the transfer of electrons from the Fe subunit to the Mo-Fe subunit. Curiously, while all substrate reductions (including CN^-, HCCH and other triple-bond compounds) occur on the Mo-Fe subunit, proton reduction (H_2 production) is unaffected by CO (13,21).

An understanding of H_2 biosynthesis by R. sphaeroides requires knowledge of the extent to which these two systems operate in vivo. Hillmer and Gest (37) have discussed the problem for R. capsulata and concluded that nitrogenase appears to be the sole enzyme responsible for H_2 biosynthesis in that organism.

CHAPTER 1. PHYSIOLOGY OF HYDROGEN PRODUCTION

INTRODUCTION

One of my initial concerns in looking at large scales H_2 production through microbial action was to find an organism which had the characteristics of easy culture, growth on a variety of carbon sources and high rate of H_2 evolution. Brown algae, blue-green bacteria, photosynthetic bacteria and certain non-photosynthetic bacteria can produce H_2 (8,31, 67,82,84). The photosynthetic bacteria show the highest rates of H_2 evolution, presumably because of the energy available to cells through photosynthesis and from their ability to grow anaerobically, an ability that protects the O_2 sensitive H_2-producing enzymes. The blue-green alga Anabaena cylindrica, for example, has been reported to produce on the order of 30 μl of H_2 per mg dry weight per hour under optimum conditions (8,84). I have observed R. sphaerodies to produce on the order of 300 l of H_2 per mg dry weight per hour at maximum. In preliminary experiments, it was observed that Rhodospirillum rubrum and R. sphaeroides could produce H_2 gas when they were fortified with lactate, malate, acetate, fructose or sucrose, and grown on a minimal nitrogen medium. They also proved easy to maintain in culture and easy to handle experimentally. Ultimately, R. sphaeroides proved to be more reproducible and the study of the physiology and biochemistry of H_2 production in this organism became the focus of this research.

According to van Niel (79), the genus Rhodopseudomonas can be separated into species based on the characteristics of the exogeneous gelatin

and mucus produced. These qualities were reported to be pH dependent. For R. sphaeroides, the gelatin is not liquified and slime formation is reported to be extensive at pH above 7. Tartrate, gluconate, ethanol, glycerol, mannitol, sorbitol, glucose, fructose and mannose can all serve as oxidation substrates. Cultures grown on sugars produce large amounts of acid, as reflected by pH drop.

With these facts in mind, it seemed appropriate to first determine a successful method for culturing R. sphaeroides, both in maintenance conditions and under conditions allowing H_2 production, and then to look at the physiology of the H_2 producing mechanism itself. Both culturing (17,55,57,70,80,86) and H_2 production (37,63,70) had been looked at in various members of Rhodospirillum and Rhodopseudomonas, but not completely so in R. sphaeroides. As part of determining culture conditions to maximize and prolong H_2 evolution, a ten liter culture was established and it maintained H_2 production for several weeks.

There are two possibilities for the enzymes that produce hydrogen during photodissimilation reactions carried out by R. sphaeroides: 1) hydrogen formation is catalyzed by nitrogenase when molecular nitrogen (the natural substrate for this enzyme complex) is not available to the cell, 2) hydrogen is formed by a completely separate enzyme system, e.g. similar to hydrogenases found in many Clostridia. Compelling evidence for either of these hypotheses is lacking, but several lines of circumtantial evidence favor the first possibility.

A number of observations obtained from studies on the physiology of the photosynthetic bacterium Rhodospirillum rubrum suggest a relationship between nitrogenase and photosynthetic hydrogen evolution. In much of the

earlier work done on this process, Gest and his co-workers (28,32,37,38,63) established the following facts:

a. ammonium ion strongly represses the photosynthetic production of hydrogen,

b. molecular nitrogen inhibits photosynthetic hydrogen biosynthesis; and

c. hydrogen occurred only in cells which were growth limited for ammonium ion.

From these results, these authors have suggested the possibility that the nitrogenase and hydrogenase activities might be catalyzed by the same enzyme (32,37). I have confirmed these observations with R. sphaeroides.

In subsequent work on regulation of nitrogenase biosynthesis in R. rubrum, it was shown that induction (or derepression) of nitrogenase occurred under exactly the same conditions used to elicit photosynthetic hydrogen production (32). I have shown here that the specific activities of nitrogenase and hydrogenase increase in parallel when cells are grown under conditions which permit different degrees of nitrogenase induction (e.g., growth on different substrates and at different concentrations of ammonium ion). Finally, it is now well established that purified nitrogenase is also a potent hydrogenase in the presence of ATP and a suitable electron donor (21,82). Moreover, crude enzyme preparations of Azotobacter vinelandii evolve hydrogen gas (if provided with ATP and an electron donor) if the cells are previously induced for nitrogenase, but not if the cells are grown in excess ammonium ion which represses nitrogenase biosynthesis (74,88). In the same study, auxotrophic mutants of A. vinelandii which

lacked nitrogenase due to loss of one of the structural genes required for an active enzyme complex, did not form hydrogen, regardless of the presence or absence of ammonium ion in the growth medium of the cells. In this section, I show that when hydrogenase is inhibited with carbon monoxide, H_2 evolution continues. Thus, the weight of evidence from several bacterial systems favors nitrogenase as the catalyst for hydrogen production.

MATERIALS AND METHODS

Organism. Wild-type *R. sphaeroides* was obtained initially from R. Stainer.

Media and Culture Conditions. Cells were grown and maintained in 15 ml screw cap vials in a modifier Hutner's medium of 2% (v/v) Hutner's concentrated mineral base with growth factors (16), 1% (v/v) yeast extract (Difco), 5 mM Na_2HPO_4, 5 mM NaH_2PO_4 in glass-distilled water, pH 7.2 ("rich" medium), fortified with 10 mM of the particular carbon source required. The experimental ("minimal") medium was as above except that 0.2% (w/v) yeast extract or experimentally defined levels of NH_4Cl or $NaNO_3$ were provided as the fixed nitrogen source. The carbon source was provided at 10 or 20 mM. In all cases, cells were grown anaerobically under illumination from a bank of G.E. "lumiline" incandescent bulbs in a temperature-controlled light box. Temperature was maintained at 27-28°. Light intensity was measured with a Weston illumination meter, model 756. Cell density was measured with a Klett-Summerson photoelectric colorimeter, model 800-3 with a red (660 nm) filter. Protein was determined by the method of Lowry *et al.* (53).

Measurement of Gas Production. Cells were grown in three types of vessels, depending on the experiment: (i) When a gas phase was not necessary and only H_2 production was of interest, cells were grown in 25 ml test tubes fitted with a 0.01N gas trap (Fig. 1-1). The tubes were completely filled with minimal medium. Gas in the needle prevented back flow of NaOH into the culture medium. Evolved gas collected at the top of the trap. The volume of this gas was measured by drawing the gas off into a calibrated, gas-tight syringe. (ii) When a gas phase for the system was necessary, 100 ml bottles with attached traps and pipettes were used (Fig. 1-2). Flasks contained 50 ml minimal medium and a gas phase of known volume, 50-70 ml. The trap contained 0.1 NaOH. Gas evolved by the cells was measured manometrically with the attached calibrated pipette. Gas was sampled through a serum stopper above the gas phase. In the third system (iii), cells for hydrogen production were grown in Hungate-type tubes (Belco) equipped with flange-type butyl-rubber stoppers to allow sampling of the gas phase above the cultures or the contents of the liquid medium. All gas samples were withdrawn with a gas-tight syringe; liquid samples were taken with 1 ml glass tuberculin-type syringes. In all cases, sampling was carried out aseptically. It was determined that over a period of several hours the serum stoppers did not allow loss of a measurable amount of H_2 in any of the three systems.

To begin the experiment, cells were inoculated with 1-5% by volume of a stationary culture, and the flasks were assembled and flushed with the appropriate atmosphere. This flushing was carried out by passing a stream of the purified gas through the vessels via two stainless steel needles. The injection needle was connected to a high pressure tank and both the

injection and exhaust needles were sterilized before the gas exchange was carried out. During the gas exchange, the gas stream was passed through a system of sterile cotton filters to reduce the chance of contamination of the culture. After several minutes of gassing, the intake or injection needle was rapidly withdrawn and then the exhaust needle was rapidly removed. This allowed equilibrium of the gas to approximately atmospheric pressure.

For all systems, the constituents of the collected gasses were determined qualitatively and quantitatively by gas-liquid chromatography, using a modified Aerograph 1520 gas-liquid chromatograph with columns of Poropak R, Poropak T or a 5Å molecular sieve, with peaks integrated with a Hewlett-Packard 18652 AA/D converter or using a Hewlett-Packard 5830A gas-liquid chromatograph with a column of Carbosieve 101. Carrier gases were helium and nitrogen, respectively. It was found that the alkaline traps of systems (i) and (ii) removed 85-90% of the CO_2 present.

Nitrogenase Assay. Nitrogenase activity was estimated by acetylene reduction (14). Cultures in various growth states in system (ii) flasks were flushed with 10% acetylene in argon for about 5 min which was long enough to be complete as shown by gas-liquid chromatograph (glc) analysis of the flask atmospheres. The cultures were incubated with gentle stirring for 15 min in the light, then gas samples of known volume were taken in gas-tight syringes and assayed by glc for ethylene as described above, using a column of Poropak T at 175° and a flame ionization detector. The culture flasks were then flushed with argon for 5 min. Nitrogenase activity and the long-term effect of acetylene, CO and CO_2 on the production of H_2 gas

were measured using the modified hungate tubes (system (iii)). Ethylene production was determined by glc as described above with a Hewlett-Packard 5830a gas-liquid chromatograph. Samples in these experiments were with-Drawn directly from above the nitrogen-limited cultures as described above.

Continuous-culture System. An 8" plexiglass tube 3 1/2' high and closed at both ends was constructed as a growth vessel (Fig. 1-3). Ports with serum type stoppers were constructed to allow access to both atmosphere and medium. Fittings to allow the attachment of a recirculating pump and a gas-liquid chromatograph were mounted at the upper end of the tube. Illumination was provided by two 200 watt spotlights placed 6" away on opposite sides of the tube. Cooling was by two banks of 3 muffin-type fans placed perpendicular to the light source. A magnetic stirrer was mounted on the bottom of the flask.

RESULTS

Maintenance Cultures. *R. sphaeroides* was initally grown on Hutner's mineral base with growth factors, 20 mM Na_2HPO_4 and 1% hydrolyzed yeast. It was observed that cells grown this way required an acclimatization period of 24-60 hrs before growing when transferred to a nitrogen minimal medium fortified with a reduced carbon source. To minimize this lag period, particular reduced carbon sources such as lactate or sucrose were added to the 1% yeast extract growth medium at a concentration of 20-40 mM. While this reduced the acclimatization period to as little as 8-10 hrs, it was observed that the cells tended to clump and settle out of solution or

become very viscous after growth had reached the stationary phase. The pH of the medium in these cases was either much higher (pH 8-9) or lower (pH 3-5) than the 7.0 of the starting medium, reported by van Niel to be the optimum for growth. To minimize this, the medium was altered to 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4, pH 7.2, as the phosphate source. This alteration minimized the pH changes, clumping of cells and secretion of mucus for all substrates except glucose. Cultures with glucose as a reduced carbon source quickly became acidic and the cells settled out.

To further minimize the effect of pH change, the cultured medium was made 20 mM in HEPES (N-2-Hydroxyethel piperazine-2-ethane sulfonic acid) buffer. This additional buffering power allowed growth of the cells on glucose to high density and prevented the drop in pH.

The 15 and 25 ml screw-cap tubes used for maintenance cultures were entirely filled with medium to minimize the exposure to O_2, although the medium itself was not treated in any way to remove dissolved O_2. There was no discernable improvement in growth when the medium was treated first to vacuum, then an argon atmosphere, and kept anaerobic.

Van Niel reported that the optimum temperature for growth of wild-type R. sphaeroides is 30° (79) and the maintenance cultures for these experiments were kept at 27-29°C. It was observed that the temperature played no effect over the range 25-40°. Temperatures above 45° result in cell death.

Experimental Cultures. R. sphaeroides will produce H_2 in large amounts when grown anaerobically in the light in a medium containing a source of electrons and minimal amounts of fixed nitrogen. Therefore, the initial

culturing of these cells for H_2-production was done on the same Hutner's mineral base with 20 mM $NaHPO_4$, 20 mM reduced carbon source and only 0.2% hydrolyzed yeast. 0.2% yeast extract allowed for 4-5 doublings of the cells in the log phase of growth, and the cell density did not become so great that light could not reach all parts of the experimental vessels.

As seen in maintenance cultures grown on "rich" medium, clumping of cells and the production of mucus associated with pH changes were also observed in cultures grown on nitrogen-minimal medium. To offset this, the "minimal" medium was modified to be 10 mM NaH_2PO_4 and 10 mM Na_2HPO_4, pH 7.2, which provided sufficient buffering power to eliminate large pH changes. In later experiments requiring minimal phosphate in the medium, 20 mM HEPES buffer was substituted with similar results.

Physiology of H_2 Production. Light-dependent biosynthesis of hydrogen gas occurred in growing cultures as well as resting cultures, but it was not clear whether cellular growth is required in those cells evolving H_2. The optical density of those cells grown on nitrogen-minimal medium and a limited amount of lactic acid was seen to increase prior to the onset of H_2 production and decrease as rates of gas production fell (Fig. 1-4). Measurements of dry weight of cells show a rise in cell mass prior to H_2 production that remained essentially unchanged both throughout and subsequent to gas production.

R. sphaeroides was grown under standard conditions in type (i) culture tubes with gas traps and with minimal medium and 20 mM lactate until gas evolution was observed. The cultures were then placed in beakers filled with water, at 15°, 20°, 25°, 30°, 35°, 40° and 45° while

all other conditions remained the same. It was observed that cultures at 25°, 30°, 35° and 40° maintained rates of H_2 production similar to that at standard conditions (27-28°). Cultures at 45° stopped producing H_2 after 1-2 hours and did not resume H_2 production when returned to standard temperatures. Cultures at 15° and 20° showed a decrease in H_2 production over several hours and this decrease could be reversed by returning the cultures to standard temperature.

Although this organism is able to utilize oxygen to some extent for growth in the dark, light appeared to be necessary for both the initiation and the continuation of H_2 production *in vivo*. Cultures of *R. sphaeroides* were grown on minimal medium with 10 mM lactate and various light intensities under standard conditions. Dark grown cultures failed to begin producing hydrogen. Low light intensities led to longer incubation times before the onset of gas production. Higher light intensities significantly reduced that incubation time (Table 1-1). In another experiment, cultures were grown until hydrogen production rates stabilized, then shifted to various light intensities. The rate of H_2 production proved to be a function of light intensity (Fig. 1-5). Such cultures placed in the dark ceased evolving hydrogen. As light intensity increased, the rate of H_2 evolution increased. At high light intensities (>40,000 lux), rates fell off markedly.

Effect of Nitrogen Source. Growth and the production of H are affected by the nature of the fixed nitrogen source provided by the culture medium. It has been assumed that the production of H_2 begins when the culture has exhausted its supply of fixed nitrogen and growth has

stopped (57,58). This is not always the case. Cultures grown with 5 mM glutamate, aspartate, glutamine, asparagine, glycine or alanine began producing H_2 while the amino acid concentration was 1-2 mM. As this concentration generally remained constant throughout H_2 production along with the amount of cell material, it suggested that nitrogen limitation is not the only factor initiating H_2 production. Also, it was observed that cultures grown on 1% hydrolyzed yeast or vitamin-free hydrolyzed casin could produce some hydrogen after as little as 20 hrs of growth, before growth had ceased or the fixed nitrogen had been exhausted.

Ammonium and nitrate had marked effect on growth and H_2 production. R. sphaeroides was grown in minimal medium and 20 mM lactate with various levels of NH_4Cl or $NaNO_3$ as sole nitrogen sources. Culture atmospheres did not contain N_2. It was observed for ammonium ion that as growth proceeded, the ion was depleted (Fig. 1-6a). Comparatively high (20 mM) or low (0.5 mM) levels of NH_4Cl did not support or only poorly supported growth and did not support H_2 production (Figs. 1-6b, 1-7). The optimum initial NH_4Cl level for growth of cells was observed to be between 2-10 mM. However, the concentration of NH_4Cl fell to very low levels (i.e., <0.5 mM) before H_2 production was initiated. If NH_4Cl equivalent to 1 mM was added to cultures producing H_2, production was immediately inhibited. Cultures grown with $NaNO_3$ concentrations from 2-200 mM showed 60% inhibition of H_2 production at 10 mM, yet no greater than 85% inhibition at 200 mM. Nitrate did not appear to inhibit growth at any concentration.

Enzymatic Source of H_2. Some of my initial experiments on culturing R. sphaeriodes to produce H_2 examined the effect of different atmospheres

with regard to the supression or stimulation of gas production. Also, the enzymatic source of H_2 in R. sphaeroides was unknown and it became clear that manipulation of the atmosphere could shed light on this problem.

The reduction of acetylene to ethylene is one test for the function of nitrogenase. Cultures of R. sphaeroides that were actively producing H_2 were given atmospheres of 10% acetylene in argon and assayed for ethylene production. The culture atmospheres were then returned to 100% argon to allow H_2 production. This was repeated several times. It was observed that the rate of H_2 production was linearly related to the reduction of acetylene (Fig. 1-8). This was true for each of the three carbon sources employed (malate, lactate and glucose).

In a second set of experiments, cultures that were producing H_2 were given atmospheres of argon, nitrogen, acetylene in argon or acetylene in nitrogen. As can be seen (Table 1-2), H_2 production was maximal under the inert atmosphere of argon. As little as 5% acetylene in argon was sufficient to diminish the H_2 evolution rate to about one-half; 50% acetylene inhibited H_2 production completely. An atmosphere of 50% argon-50% nitrogen inhibited the acetylene reduction assay slightly, but lowered H_2 production to about 30% of the control level. 95% nitrogen-5% acetylene suppressed H_2 production completely. 100% nitrogen inhibited acetylene reduction by about two-thirds and a small amount of H_2 production remained. An atmosphere of 100% H_2 did not appear to affect acetylene reduction or H_2 production.

In further experiments, cultures actively producing H_2 were treated to atmospheres of either 10% carbon monoxide in argon or 18% O_2 in argon.

The effect of carbon monoxide (Fig. 1-9) was to suppress acetylene reduction completely, but the rate of H_2 production was only slightly, if at all, reduced by this gas. This effect proved to be reversible. When CO was removed from cultures showing inhibition, acetylene reduction activity generally returned within one to two hours. After treatment with O_2, all H_2-evolving and acetylene-reducing activity was lost after a few hours and did not return when the experimental flasks were flushed with argon to remove O_2 (Fig. 1-10). The cells were not dead, as they could be recultured in fresh medium to produce H_2. It should be noted that cultures 4% in O_2 did not show significant inhibition of H_2 production or acetylene reduction.

Large-scale Studies. It was observed that the rates of H_2 production in any particular culture initially rose to a maximum, leveled off and eventually fell (Fig. 1-4). Sometimes, the cultures maintained a low, endogenous level of H_2 production; sometimes H_2 production ceased completely. When such cultures were given more of the appropriate reduced carbon substrate after gassing had slowed, it was observed that the rate of H_2 production rapidly increased, often to greater levels than were seen initially (Fig. 1-11). This replenishment of reduced carbon source could be repeated several times before the cell ceased producing H_2 altogether.

A series of cultures was brought to H_2 production on malate and received additional malate every other day. At one to two week intervals, additions of hydrolyzed yeast and/or concentrated Hutner's mineral base were added to restore amino acids and trace elements to the media. It was observed (Fig. 1-12) that cultures receiving only malate as replenishment produced less than 10% of initial rates after 38 days, while cultures also

receiving mineral base maintained 30% of the initial H_2 production after that length of time. Cultures fortified with yeast extract, or yeast extract and mineral base maintained 50% and 80% respectively of initial rates after 38 days and continued to do so until the end of the experiment.

Cultures were grown in type (ii) flasks on 10 mM malate with either Ar or 90% Ar plus 10% N_2 as atmospheres. All flasks were resupplied with the equivalent of 10 mM malate every other day. It was observed that flasks containing 10% N_2 in the atmosphere maintained up to 80% of the initial rates over four weeks, while the control flasks on Ar alone fell to 25% of initial rates after that period of time.

To see if these operations would hold for larger scale operations, a ten liter continuous culture vessel was prepared with minimal medium and 20 mM lactate as the reduced carbon source. R. sphaeroides was maintained therein for six weeks by addition of lactate every other day and concentrated Hutner's base and yeast extract every week. Growth was heavy even in 0.2% hydrolyzed yeast and the cultures had to be continuously stirred throughout the experiment. After 36 hrs, H_2 began bubbling out of solution and was monitored by gas-liquid chromatography and by flame test. H_2 was produced throughout the experiment. The rate of H_2 production varied from 400-900 ml per hr. The ratio of H_2 to CO_2 in the atmosphere was more-or-less constant at 2 to 1.

DISCUSSION

Nitrogenase. The data in this paper supports the concept that H_2 biosynthesis in R. sphaeroides is mediated by nitrogenase. This view is based on several experimental lines of evidence. First, acetylene and molecular nitrogen, both substrates for reduction catalyzed by nitrgenase, inhibited H_2 evolution. As might be expected, increasing the percentage of these gases in the cultures exposed to these compounds increased the inhibitory effect. Second, ammonium ion, which is generally a repressor of nitrogenase synthesis (58), blocked the synthesis of H_2. Third, the cellular specific activity of nitrogenase was coordinately (linearly) related to the specific activity of the H_2 producing enzyme. And, finally, the effects of CO and O_2 on in-situ H_2 biosynthesis were predictable, based on published results with the purified enzyme (21,40). As reported by others, CO blocks nitrogenase catalyzed reduction of molecular nitrogen without inhibiting H_2 evolution (40), while O_2 causes the loss of both the H_2 biosynthesis and N_2 reduction activities. We have demonstrated here a similar effect for R. sphaeroides.

It appears that acetylene is a better substrate for nitrogenase than are protons, since acetylene can completely suppress H_2 production. N_2 must be less effective, since some H_2 production occurs even at 100% N_2 (Table 1-2). An atmosphere of 100% H_2 does not appear to affect H_2 production, suggesting that the reuptake of H_2 is of little consequence.

After treatment with carbon monoxide, acetylene reducing capacity was lost from cultures although hydrogen biosynthesis was not significantly affected. Control cultures showed no change. It should be noted that when carbon monoxide is removed from cultures showing inhibition,

acetylene reduction generally returns after about an hour. It could be argued that this is an assay of an *in vivo* system and that the results could be caused by carbon monoxide affecting other functions. For example, adenosine triphosphate (ATP), which is a required substrate for nitrogenase, but not for hydrogenase, might be diverted or not synthesized. Assuming that the intracellular concentration of carbon monoxide is too low to inhibit the cytochrome system for hydrogenase, the data could be explained by an active hydrogenase and a nitrogenase that is not producing hydrogen. For this case, two strong rebuttals exist. Both nitrogenase and hydrogenase appear to require reduced ferredoxin as the primary electron donor. In this case it would be competition for ferredoxin between nitrogenase and hydrogenase, so that when nitrogenase was inhibited, we would expect an increase in hydrogen production. This is not observed. Also, although hydrogenase does not require ATP directly, ferredoxin is reduced in the cell by an ATP requiring reaction. Therefore, if ATP synthesis is affected by carbon monoxide, hydrogen production should cease altogether. This has not been observed. Historically, hydrogenases and nitrogenases have been found to be closely associated, both spatially and structurally, in organisms having both enzymes. It seems probable, then, that if carbon monoxide does not show the same effects towards these enzymes *in vivo* as *in vitro*, then hydrogen probably is produced by an unknown, third system.

All hydrogen-evolving and acetylene-reducing activity was lost over a period of a few hours from those cultures receiving O_2. The controls were unchanged. This loss of activity was not regained when the experimental flasks were flushed with argon to remove O_2. This supports the idea that

nitrogenase is the functioning enzyme for hydrogen production in R. sphaeroides. Nitrogenase, by this hypothesis, is destroyed. Resynthesis does not occur since the system was originally under limiting fixed nitrogenase and this presumably has been exhausted prior to the onset of the initial enzyme activity. The fact that addition of fixed nitrogen in the form of yeast extract after O_2 inhibition yields renewed hydrogen evolution and acetylene reduction is somewhat equivocal. It is possible that this reuslts from growth of new cells rather than resynthesis of enzyme.

Effect of Fixed-nitrogen Sources. While ammonium suppresses H_2 biosynthesis completely and nitrate ion inhibits it, fixed nitrogen in the form of amino acids seems to have little effect in H_2 production. This allowed the continuous culturing of R. sphaeroides in a mode that yielded H_2 gas. This process required light and a reduced carbon source. While high levels of N_2 in the atmosphere inhibited H_2 production, 10% N_2 helped maintain rates of H_2 production by supporting cells viability. Since ammonia is the reduction product of N_2, it appears that ammonium and nitrate rather than amino acids are regulators of nitrogenase.

TABLE 1-1. Initiation of H_2 Production as a Function of Light Intensity

R. sphaeroides was grown in minimal medium with 0.2% yeast extract as nitrogen source and 10 mM lactate as carbon source (pH 7.2). Cultures were anaerobic with the indicated light intensities.

Illumination	Time Before H_2 Initiation
dark	no H_2
1000 lux	70-100 hrs
2000 lux	36-48 hrs
4000 lux	16-24 hrs
8000 lux	10-17 hrs

TABLE 1-2. Effect of Inhibiting Atmosphere on H_2 Production

R. sphaeroides was initially grown under Ar in minimal medium with 10 mM malate in the light. After the onset of visible gas production, cultures were given the appropriate atmosphere. For the acetylene reduction assay, the atmospheres were made 10% in C_2H_2 for the period of the assay (15 min) and then flushed with the appropriate atmospheres. Cultures were continuously stirred to maximize the surface area of the media.

Atmosphere	Acetylene Reduction[a]	H_2 Production[b]
100 Ar	100	4.1
95% Ar- 5% C_2H_2	--	1.7
90% Ar- 10% N_2	96	3.9
50% Ar- 50% C_2 H_2	--	0.0
50% Ar- 50% C^2H_2	82	1.5
95% N_2- 5% C_2H_2	--	0.6
100% N_2	37	0.6
100% H_2	102	4.0

(a) Values indicate relative percent C_2H_2 reduced per hr per gram of protein.

(b) Values indicate milliliters of H_2 evolved per hr per gram of protein.

FIGURE LEGENDS

1-1 25 ml culture tube with attached CO_2 trap. Trap is filled with 0.01N NaOH.

1-2 100 ml culture flask with attached CO_2 trap. Trap is filled with 0.1N NaOH.

1-3 15 l culture vessel for continuous culture.

1-4 Growth and H_2 production by wild-type R. sphaeroides. Cultures were grown on minimal medium and 10 mM lactate anaerobically at 8000 lux illumination. Symbols: O-O, H_2 evolution; □-□, density; △-△, optical density.

1-5 Comparison of H_2 production with light intensity. Wild-type R. sphaeroides was grown anaerobically on minimal medium and 10 mM lactate in the light until H_2 rates stabilized, then shifted to the indicated light intensities. Rates were the average of 10 hrs under the experimental conditions.

1-6 Effect of ammonium ion on growth of wild-type R. sphaeroides. Cells were grown on minimal medium with 20 mM lactate and the indicated initial levels of NH_4Cl anaerobically in the light. Symbols: ▲-▲, 0.5 mM NH_4^+; ■-■, 6.0 mM NH_4^+; □-□, 10 mM NH_4^+; ●-●, 20 mM NH_4^+. (A) Ammonium ion levels vs. time. (B) Optical density vs. time.

1-7 Effect of ammonium ion on initiation of H_2 production. Conditions and symbols were as for Fig. 1-6.

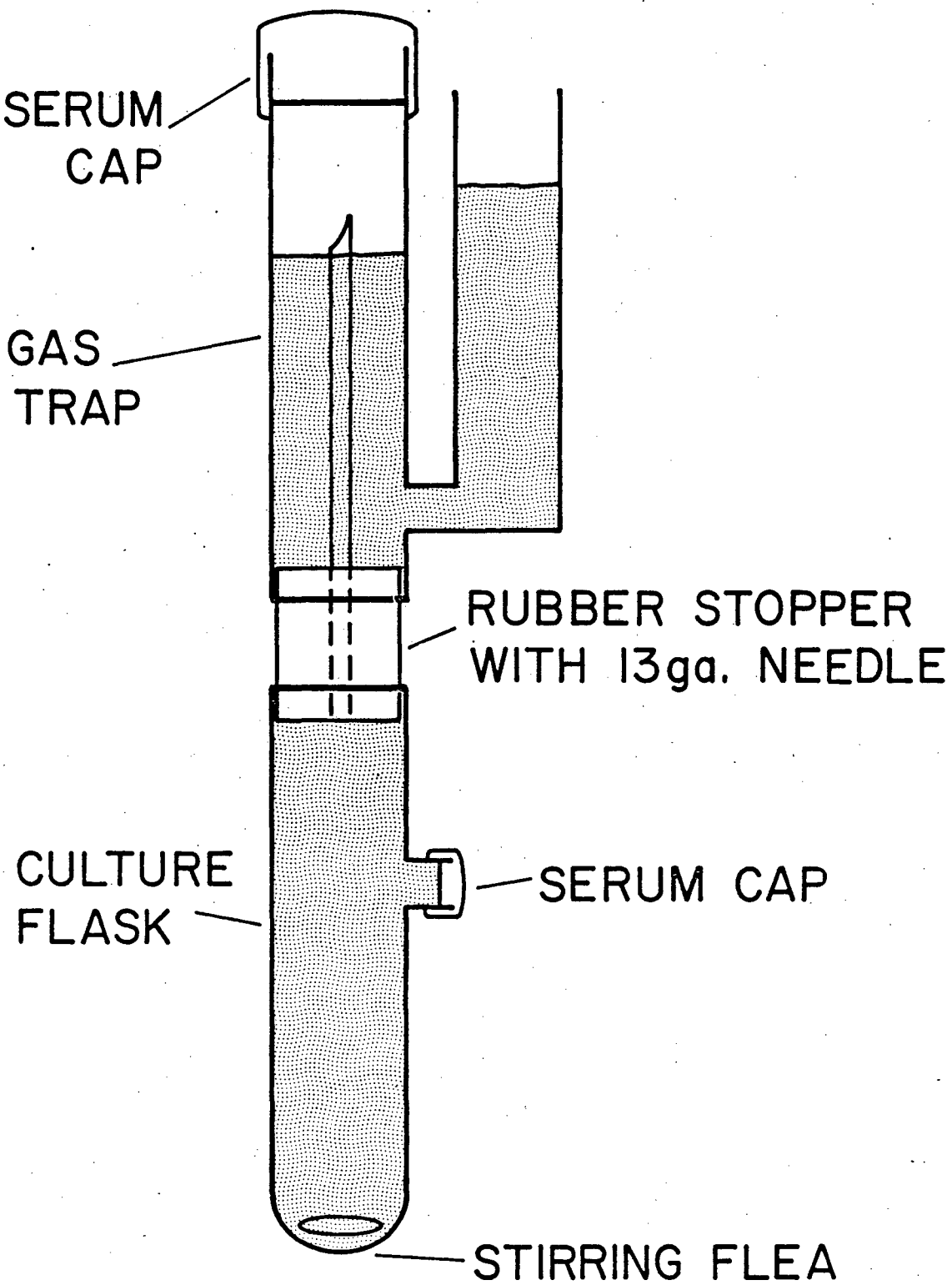
1-8 Comparison of H_2 evolution with acetylene reduction. Wild-type and Glc$^+$ mutants were grown anaerobically on minimal medium with 20 mM of the indicated carbon sources in the light. Acetylene reduction was measured by flushing culture atmosphere with 10% acetylene in argon, incubating for 15 min and assaying atmospheres for ethylene by gas-liquid chromatography. Culture atmospheres were returned to 100% argon and subsequently assayed for H_2 by gas-liquid chromatography. %/hr/g protein: Acetylene reduction is presented as percent acetylene in atmosphere reacted per hr per gram of protein. Symbols: ▲, malate, wild-type strain; △, lactate, wild-type strain; ●, glucose Glc$^+$ strain.

1-9 Effect of carbon monoxide on H_2 production. Wild-type cells were grown under Ar in minimal medium 10 mM malate in the light. After the onset of gas evolution, the atmosphere was made 10% in CO (dark bar). Assays were as in Fig. 1-8. Symbols: △-△, H_2 production, 100% Ar; ▲-▲, C_2H_2 reduction, 100% Ar; ○-○, H_2 production, 10% CO, 90% Ar; ●-●, C_2H_2 reduction, 10% CO, 90% Ar.

1-10 Effect of oxygen on H_2 production. Wild-type cells were grown in minimal medium with 10 mM malate in the light under Ar. After the onset of H_2 evolution, the atmosphere was made 18% in O_2 (dark bar). After the period under O_2, the atmosphere was flushed with Ar. Assays were as in Fig. 1-8. Symbols: △-△, H_2 production, 100% Ar; ○-○, C_2H_2 reduction, 100% Ar; ▲-▲, H_2 production, 18% O_2; ●-● C_2H_2 reduction, 18% O_2.

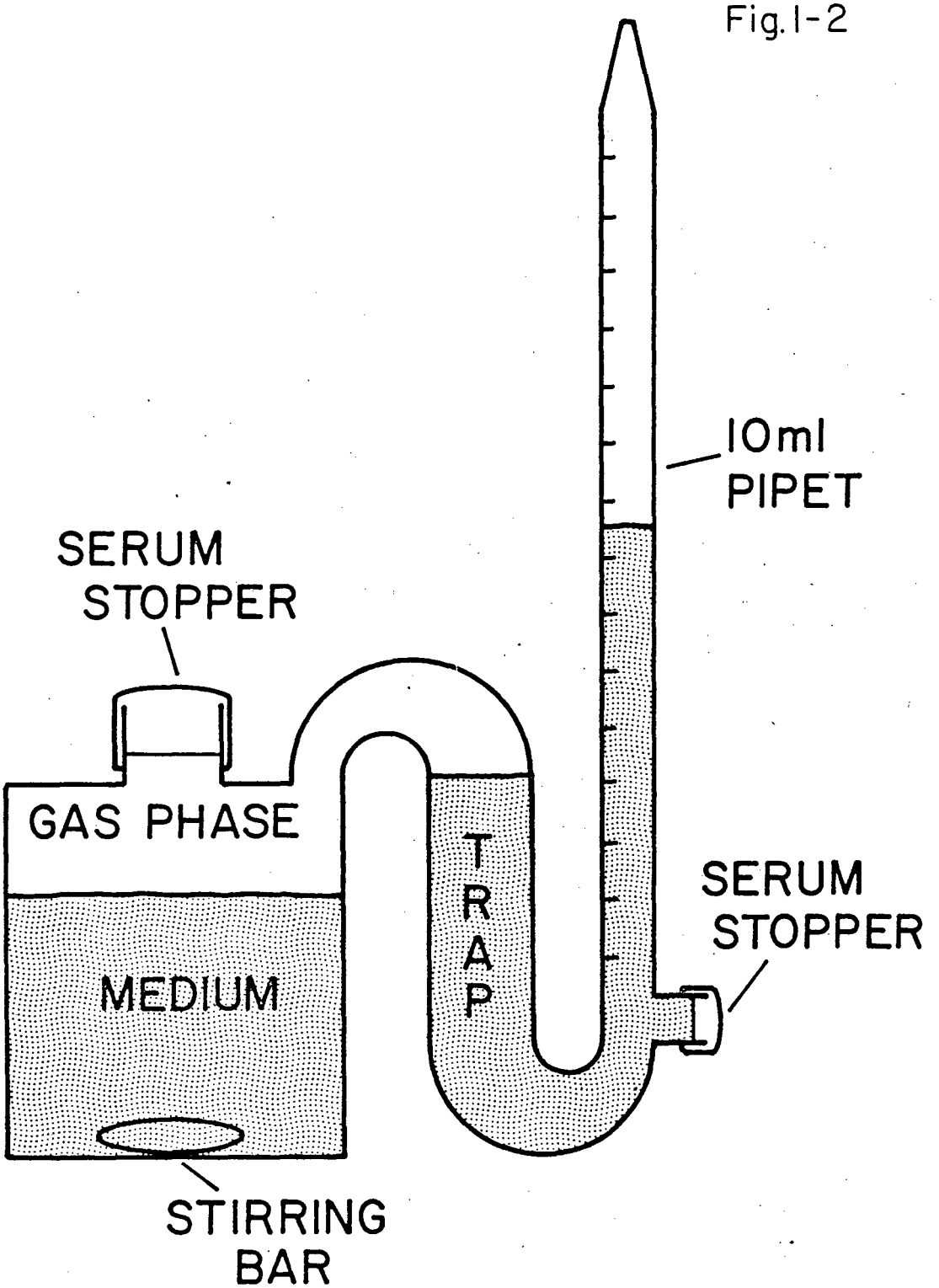
1-11 Maintenance of H_2 production rate in R. sphaerodies. Wild-type cells were grown anaerobically on minimal medium with 20 mM lactate in the light. Lactate equivalent to 20 mM concentration was added to the cultures as the H_2 evolution rate fell (arrows).

1-12 Maintenance of H_2 evolution in R. sphaeroides. Wild-type cells were grown anaerobically on minimal medium with 10 mM malate as carbon source in the light. Malate equivalent to 10mM concentration was added every other day. Replenishment of the indicated nutrients was at arrows. Symbols: O-O, no replenishment; □-□, yeast extract (0.02) added; Δ-Δ, Hutner's base (2.0%) added; ●-●, both yeast extract (0.2%) and Hutner's base (2.0%) added.


SERUM CAP
GAS TRAP
RUBBER STOPPER WITH 13ga. NEEDLE
CULTURE FLASK
SERUM CAP
STIRRING FLEA

XBL 782—3813

Fig. 1-2



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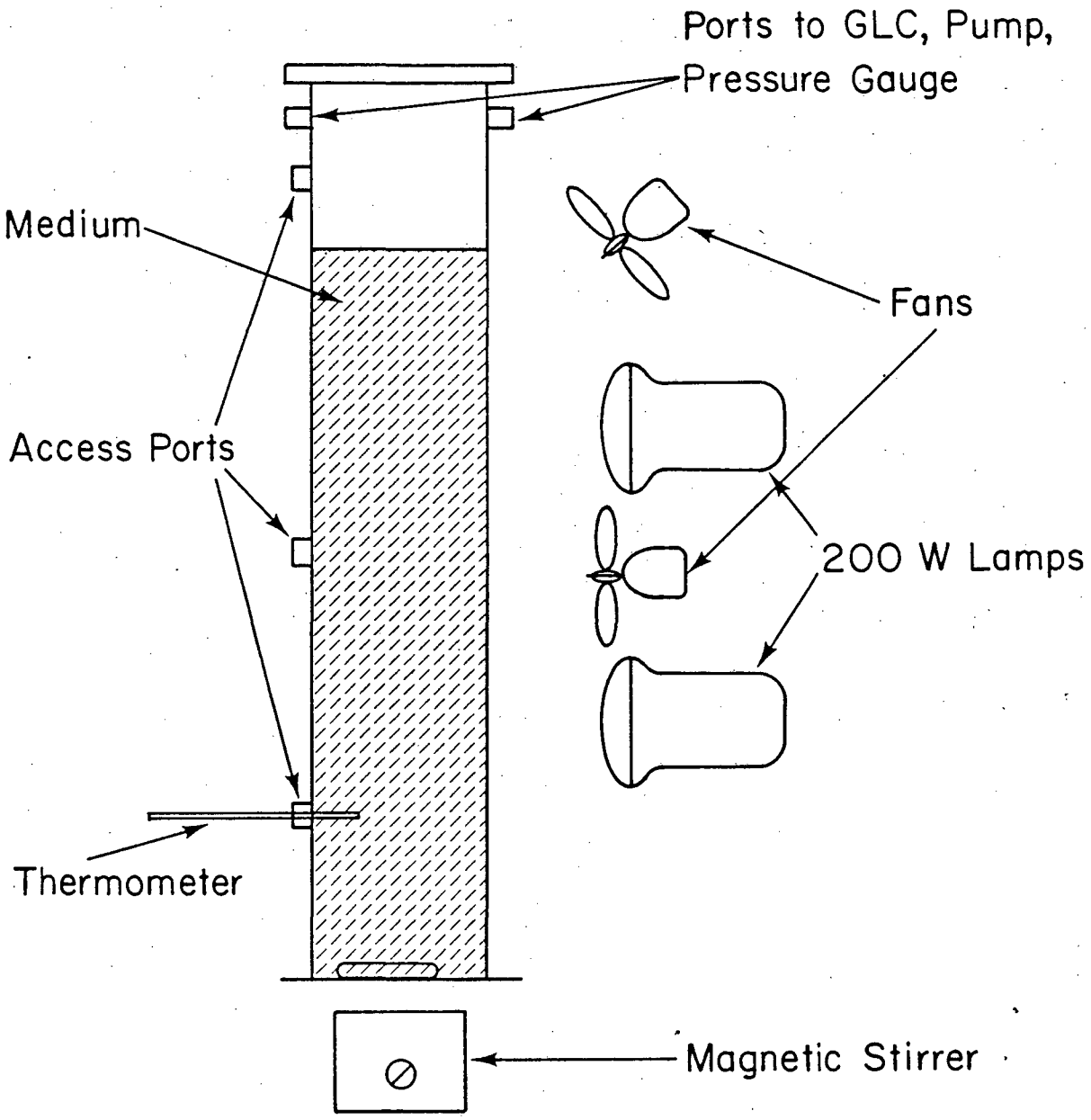
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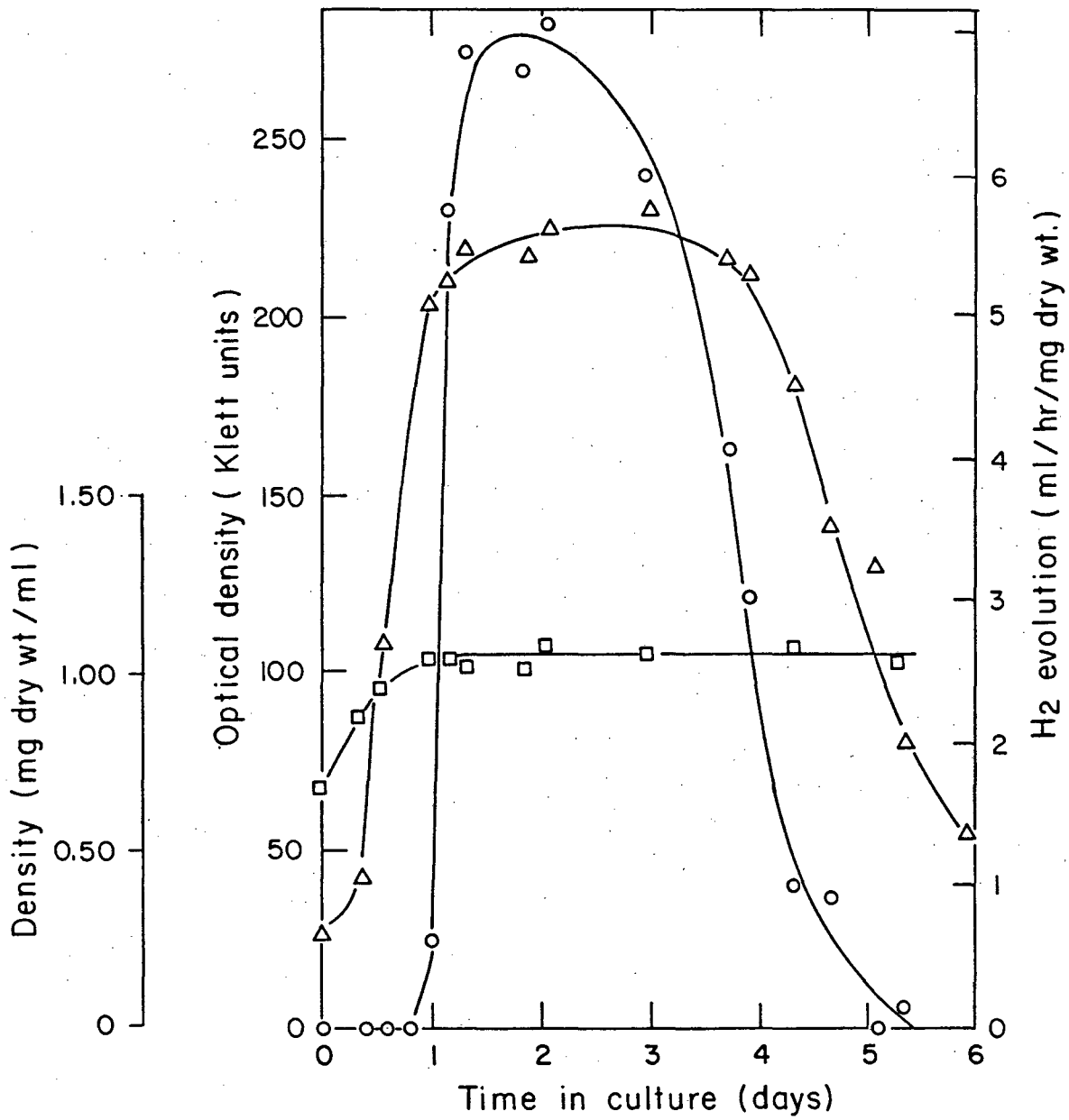
Pressure Gauge
Medium
Fans
Access Ports
200 W Lamps
Thermometer
Magnetic Stirrer

XBL 811-4402



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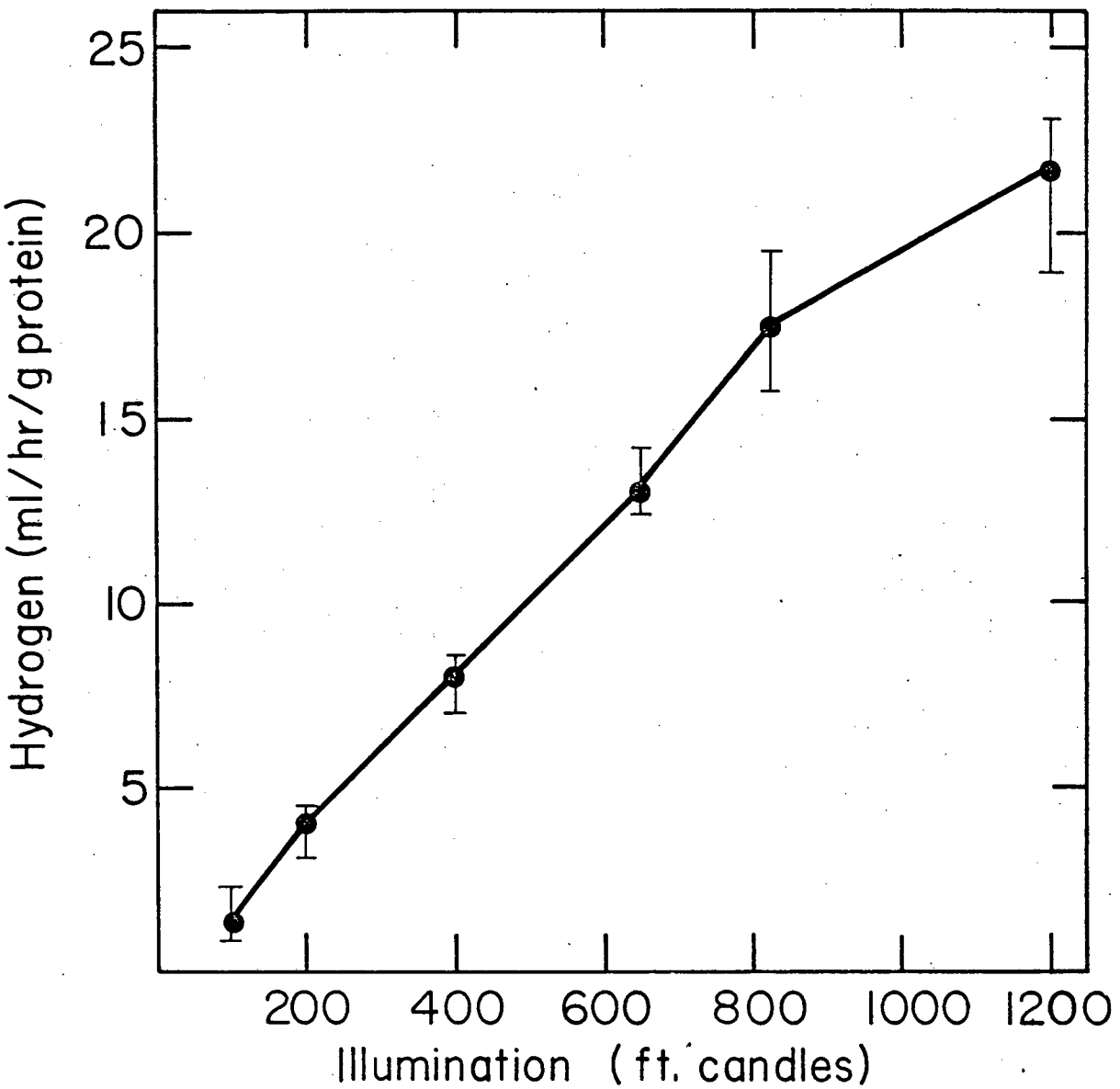
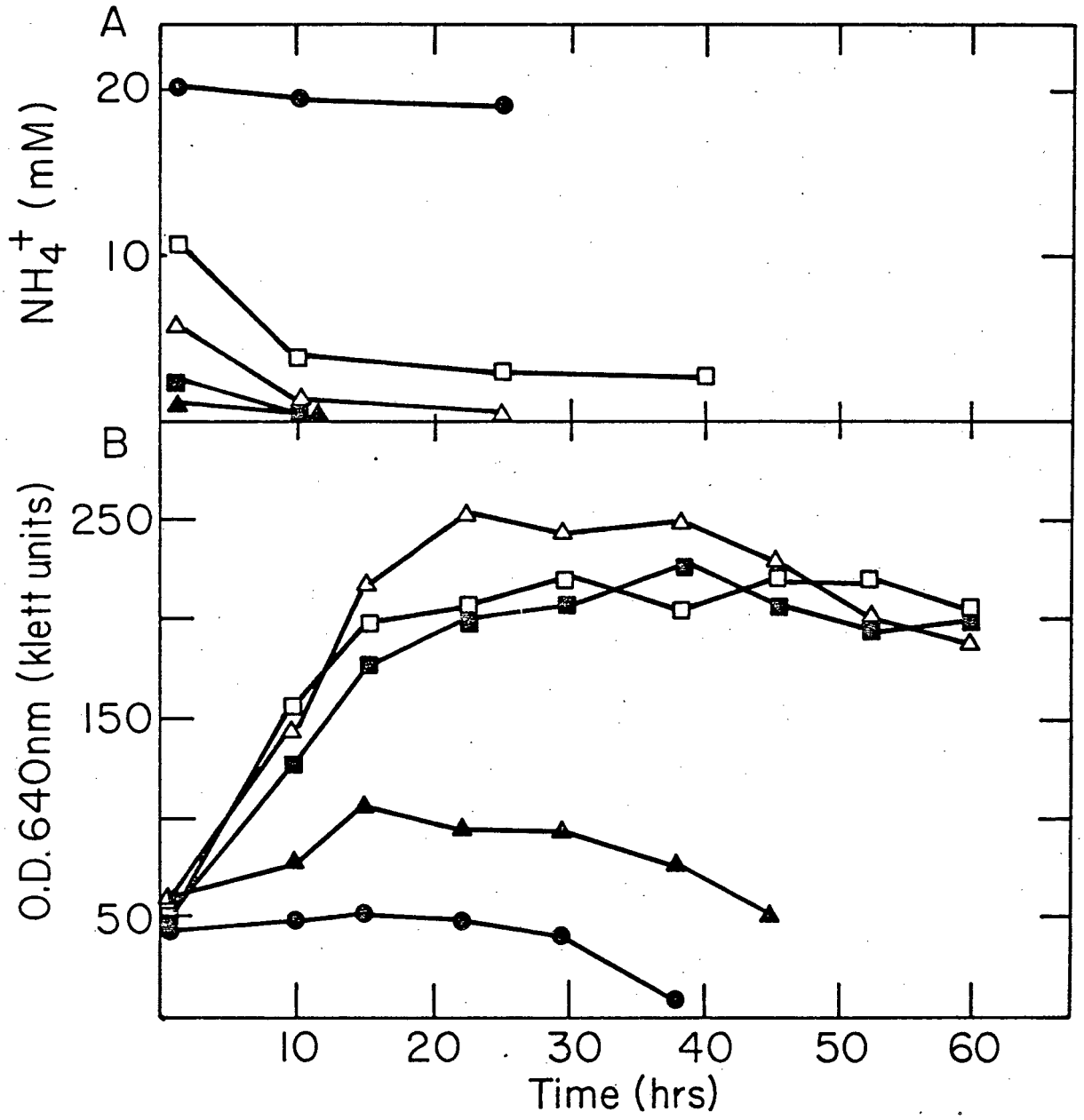
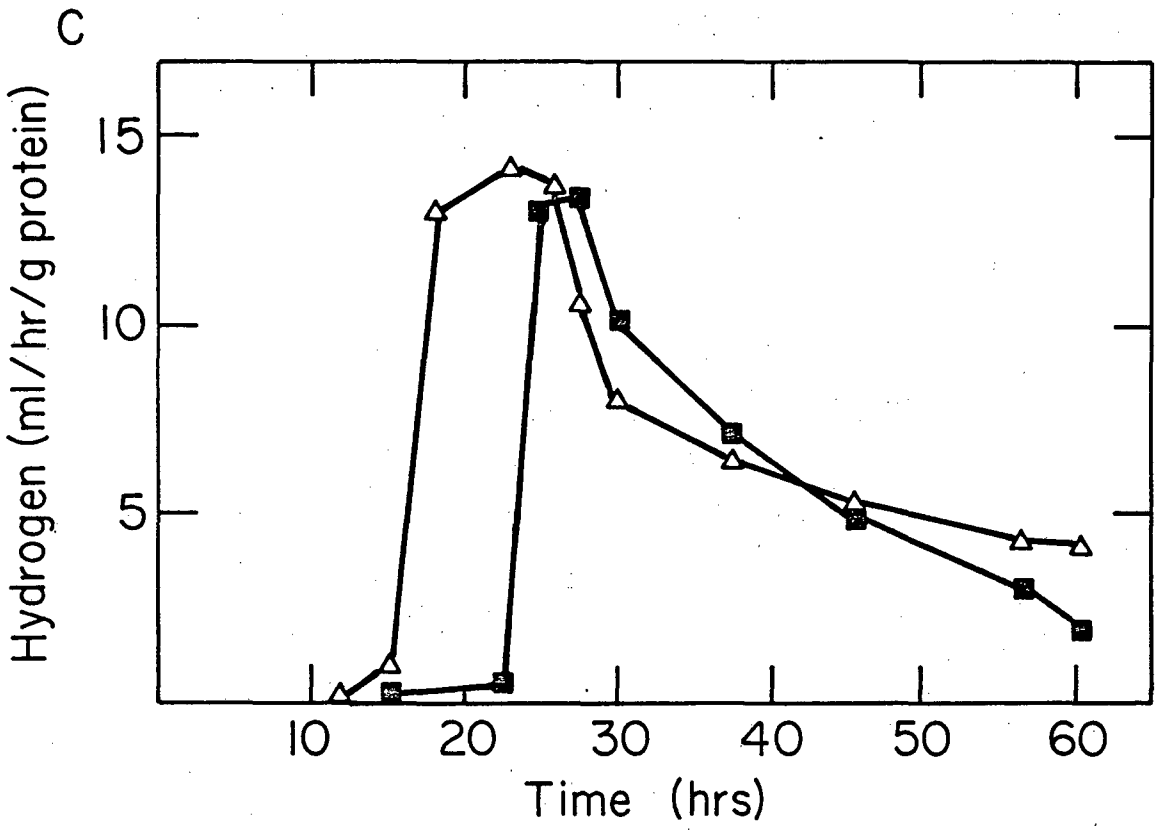

25
20
15
10
5
Hydrogen (ml/hr/g protein)
200
400
600
800
1000
1200
Illumination (ft. candles)

XBL 782-3793


A
B
20
10
NH_4^+ (mM)
250
150
50
O.D. 640nm (klett units)
10
20
30
40
50
60
Time (hrs)

XBL 789-4219



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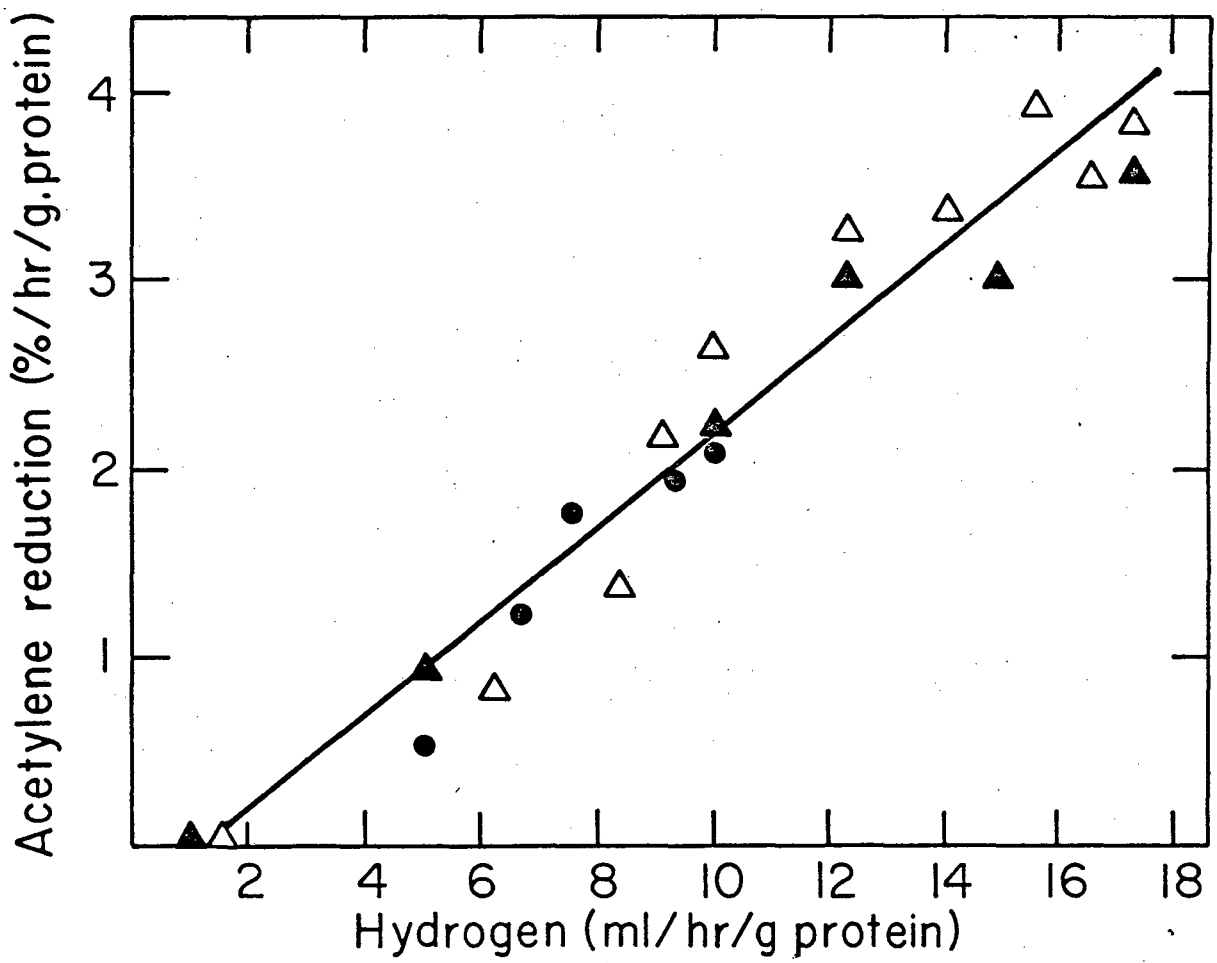
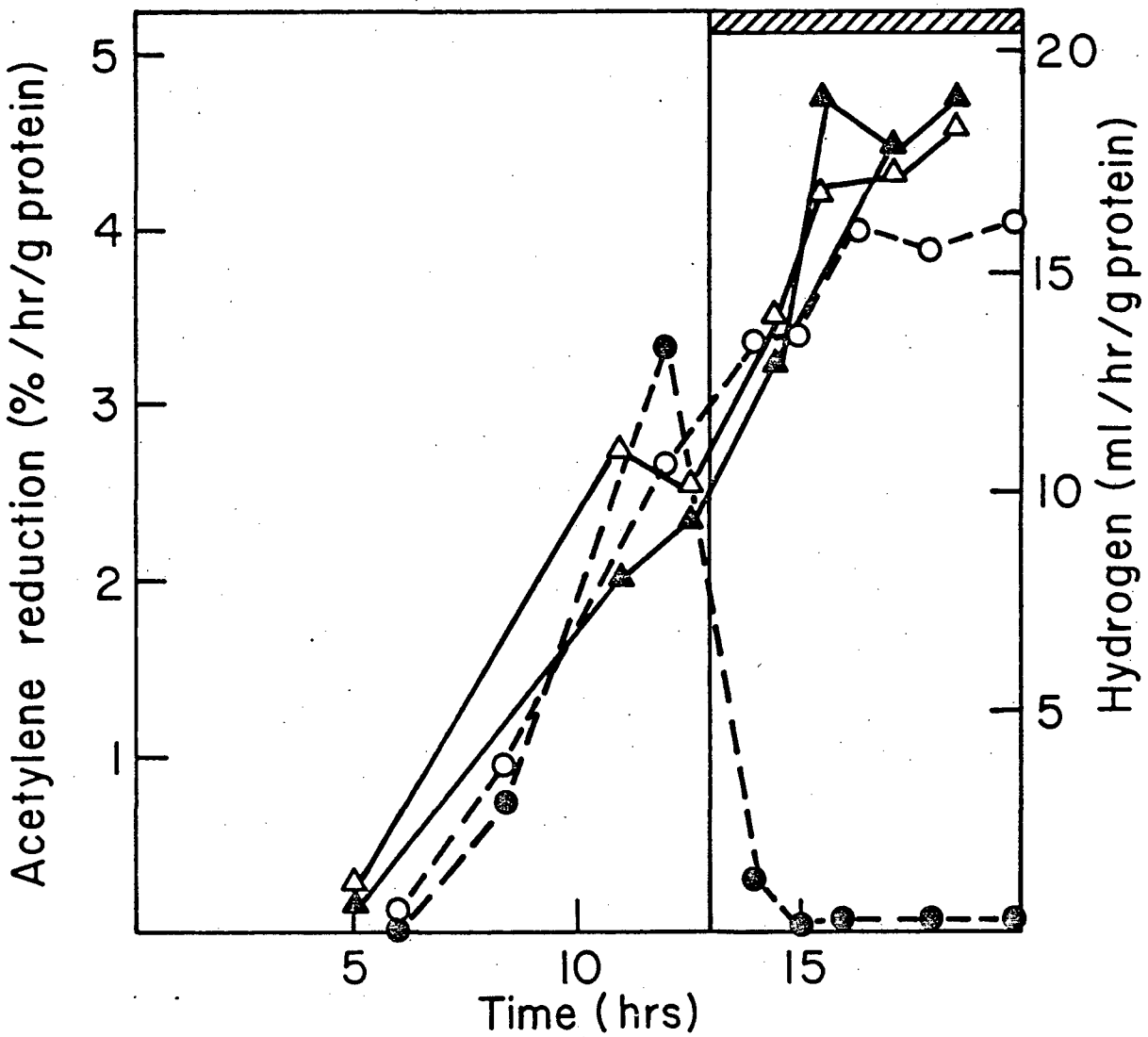
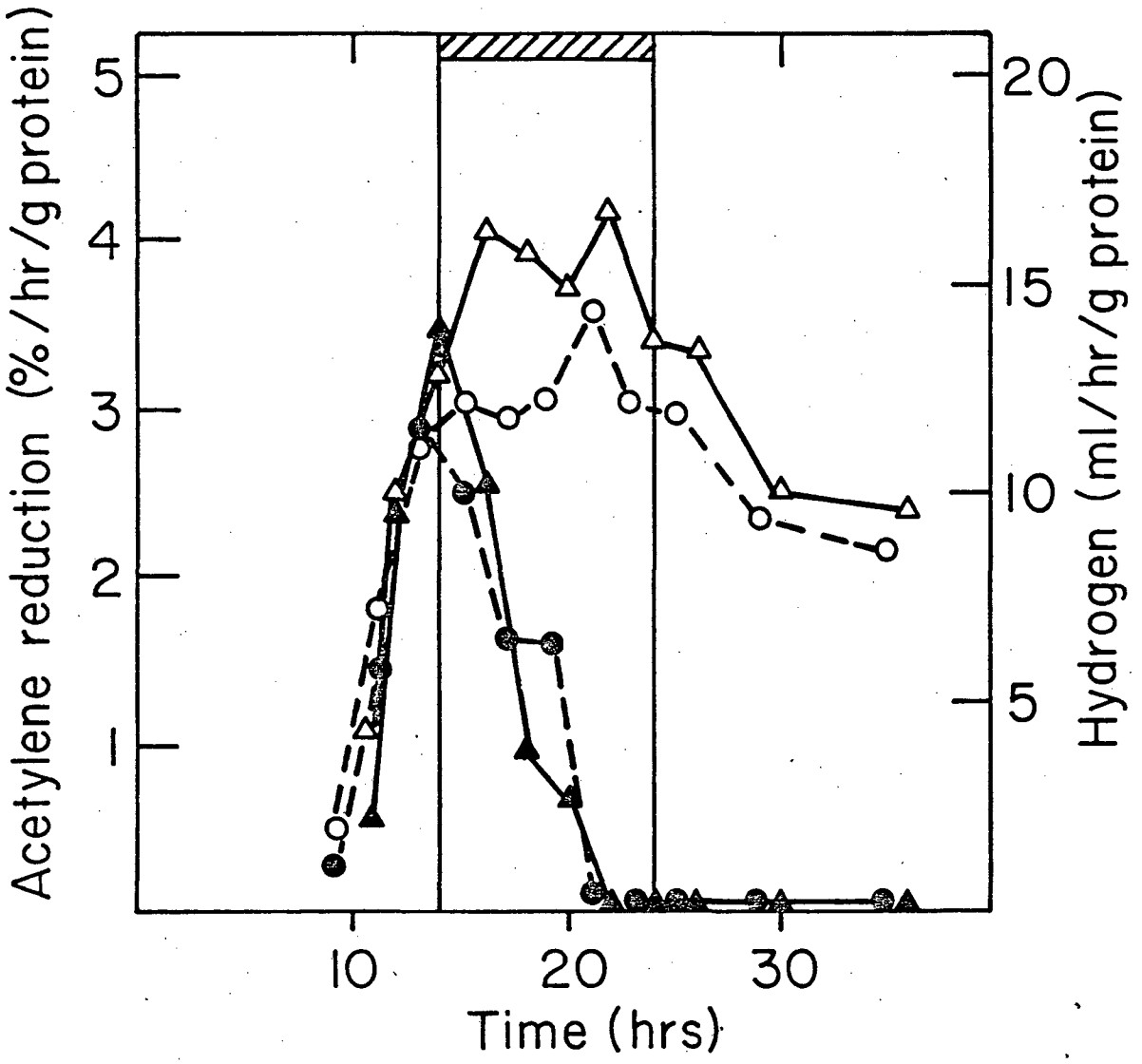

Acetylene reduction (%/hr/g.protein)
4
3
2
1
2
4
6
8
10
12
14
16
18
Hydrogen (ml/hr/g protein)

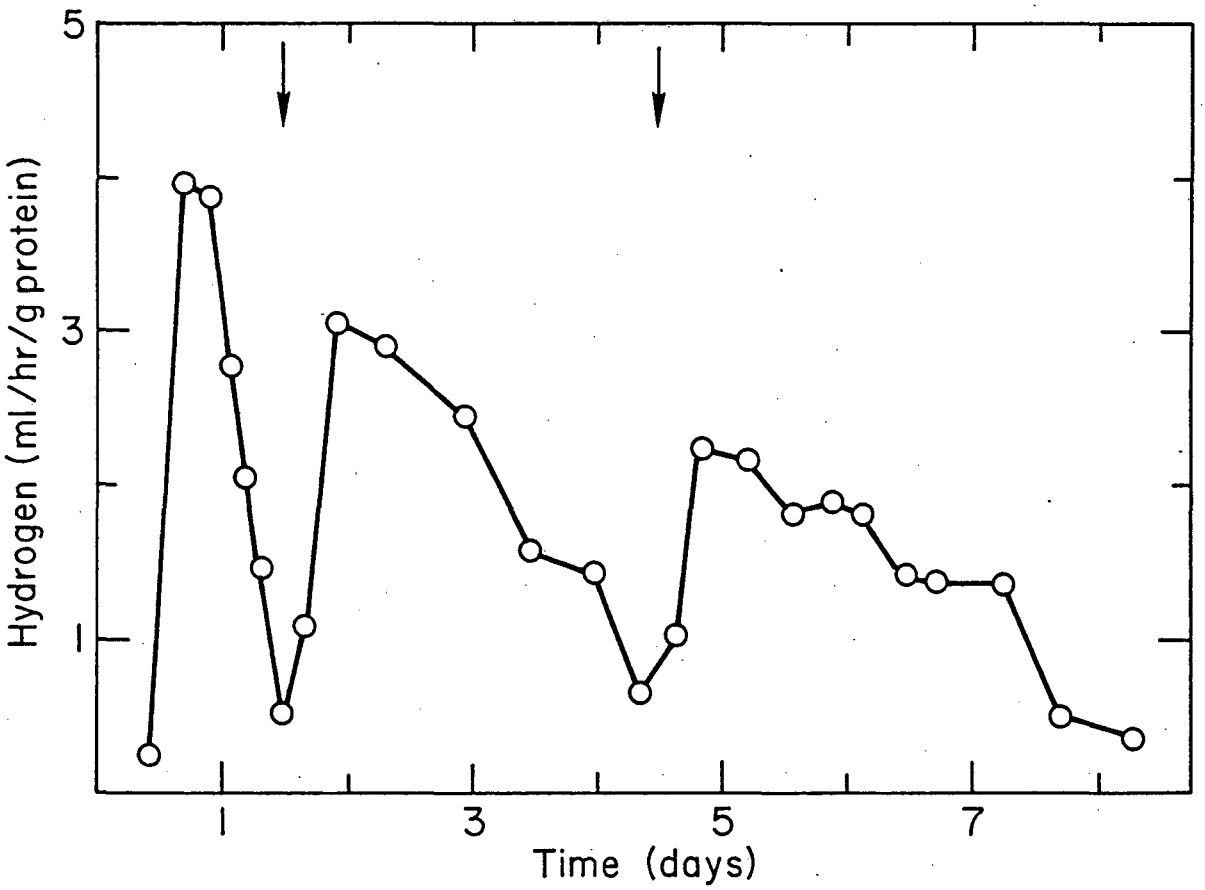
XBL789-4217


Acetylene reduction (% /hr/g protein)
5
4
3
2
1
Hydrogen (ml/hr/g protein)
20
15
10
5
5
10
15
Time (hrs)

XBL789-4216


Acetylene reduction (%/hr/g protein)
5
4
3
2
1
Hydrogen (ml/hr/g protein)
20
15
10
5
10
20
30
Time (hrs)

XBL 782-3805



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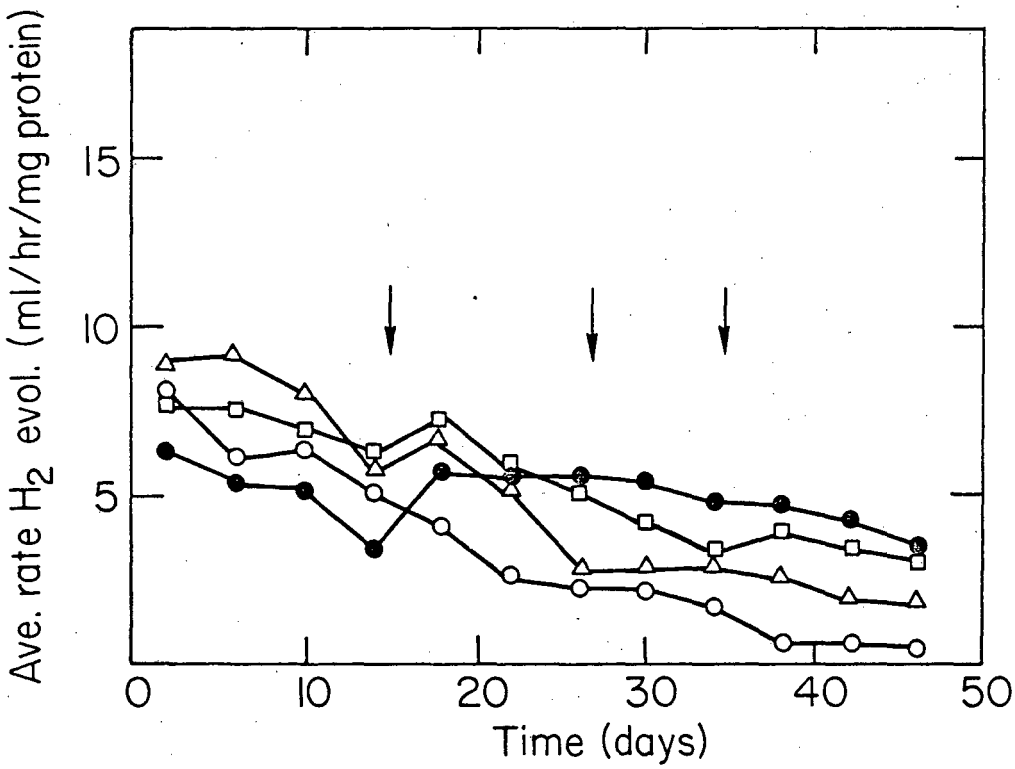

Ave. rate H_2 evol. (ml/hr/mg protein)
15
10
5
0
10
20
30
40
50
Time (days)

XBL 789-4215

CHAPTER 2. SUBSTRATE UTILIZATION AND THE GENERATION OF MUTANTS

INTRODUCTION

Van Niel reported (79) that tartrate, gluconate, ethanol, glycerol, mannitol, sorbitol, glucose, fructose and mannose can all serve as oxidation substrates for R. sphaeroides. Other substrates such as lactate, malate and acetate have also been reported as supporting growth of this organism (23,33,59,63). Sugar substrates lead to the production of acidic compounds, resulting in a decrease in the pH of the culture medium (76,79). This was confirmed in the preliminary work for this thesis.

The production of H_2 by photosynthetic bacteria has been reported to occur with lactate and malate as reduced carbon sources (63,67). It seemed useful to look further at what other compounds were suitable for H_2 production. A long-range goal of this research has been the utilization of photosynthetic bacteria for the biosynthesis of H_2, either as a primary fuel source or as a necessary component in synthetic fuel cycles (i.e., for reduction of carbon compounds containing oxygen or nitrogen). Such large-scale H_2 production would require the utilization of various kinds of reduced carbon compounds that might be readily available, such as agricultural, paper mill or sewage organic wastes. If the wild-type organism was incapable of utilizing these compounds to make H_2, it would be useful to develop or isolate mutants that were able to do this.

This section presents the data on substrate utilization by the wild-type R. sphaeroides and the development of mutant strains capable of producing H_2 gas from compounds that are utilized poorly or not at all by the wild-type strain.

MATERIALS AND METHODS

Organism and Culturing. Wild type R. sphaeroides was from the laboratory collection and was initially a gift of R. Stainer. Mutant strains capable of growth and H_2 from alternative reduced carbon sources were isolated as described in Results. Culturing of R. sphaeroides for maintenance and H_2 production was as described in Chapter One.

^{14}C Tracer Experiments. Log-phase cells in rich medium were harvested at room temperature by centrifugation at 8,000 x g for 5 min, then resuspended in 25 ml minimal medium in the 25 ml tubes with attached traps (system (ii)). The culture medium was made 20 mM with the appropriate carbon source and the flasks allowed to incubate at 27° and 9000 lux illumination. As gas production began (10-20 hrs), low levels (0.4-0.8 mC/mole) of the appropriate uniformly ^{14}C-labeled carbon source (New England Nuclear) were added to the cultures. The gas was measured and collected at intervals and injected into serum-stoppered Warburg flasks containing 2N KOH in the center wells to trap CO_2. After gas evolution ceased the culture media were collected and placed inside Warburg flasks containing KOH in the center wells. Concentrated H_2SO_4 was added to the media. The solution in the center wells of the Warburg flasks and the liquid in the traps of the culture flasks were assayed by scintillation counting using a Packard

tri-carb liquid scintillation counter, Model 3375, and Aquasol-2 (New England Nuclear) as the scintillator.

Samples of the culture media were taken at intervals throughout the period of gas production and killed in 80% methanol. Aliquots were assayed by two-dimensional paper chromatography, autoradiography and Geiger-counting (44,66). The solvent system for the first dimension was phenol-water-glacial acetic acid-ethylenediamine tetraacetic acid (840:260:10:1 v/v/v/v) and in the second dimension, equal volumes of n-butanol-water (370:25 v/v) and propionic acid-water (180:220 v/v). Chromatograms were developed 36 hrs in each direction.

CO_2 production using system (iii) was assayed in a similar manner by quantitative liquid scintillation of $^{14}CO_2$ recovered from supernatant fractions of spent media after hydrogen production ceased. Uniformly labeled malate and glucose were used as the sources of tracer. To estimate the amount of CO_2 from $^{14}CO_2$ measured, the specific activities of the individual substrates were divided by the number of carbon atoms in the given substrate. Before the evolved CO_2 was trapped, the supernatant fractions were first treated with 0.2 ml of 3 N KOH 5 ml of culture to insure that free CO_2 was converted to carbonate-bicarbonate ions. The alkaline supernates were then added to a Warburg vessel containing NCS Tissue Solubilizer (Amersham/Searle) in the center well. 4 N HCl was then added to the main chamber of the vessel and the vessel quickly sealed with a rubber stopper. After several hours of gentle shaking to remove the last of the CO_2 from the supernatant solutions, the contents of the center well were removed with a syringe, then placed in a commercial scintillation fluid (Multisol, Isolab Inc.) and counted with an IsoCap/300 6868 liquid scintil-

lation system (Searle Analytic Inc.). The amount of quenching was estimated using an external standard method programmed for the scintillation counter.

The analysis of carbon-14 assimilation was carried out as follows: cells were washed 4 x with growth medium and aliquots were added to 0.4 ml of NCS solubilizer, mixed thoroughly and added to Multisol for counting by liquid scintillation corrected for quenching.

GLC Analysis of Sugars and Sugar Acids. For certain experiments carried out with system (iii), glucose was determined colorimetrically by Glucostat reagent (Worthington Biochemical Corp.) or by quantitative gas-liquid chromatography of the trimethylsilyl (TMA) derivative. Gluconate and an unknown sugar acid were also quantitatively assayed by GLC of their respective TMS derivatives. Separation of the TMS sugars was done on a column of Chromosorb W containing a liquid phase of 3% OV-1 (Supelco Inc.). The TMS sugars were measured with a flame ionization detector (FID) and quantitated against known amounts of commercially available TMS sugars (Sigma Chemical) or with TMS samples prepared by the method described by Laine et al (51). The FID detector response was linear form 0.01 to 0.10 ugm of glucose or gluconate. All measurements were made at a constant temperature of 195° with N_2 as the carrier gas.

Samples for silylation were prepared in the following way: 1-5 ml of a cell suspension was centrifuged at 10,000 x g for 10 min and the clear supernatant fraction and cell pellet were separated. The supernatant fraction was then freeze-dried under reduced pressure and silylating reagents added to the dried residue containing the nonvolatile byproducts of the spent medium. The residue was carefully mixed with the silylating reagents, scraping the sides of the test tube with a glass rod where

necessary to insure the complete mixing. The reaction mixture was then stored at room temperature for about 6 hrs to allow complete formation of the TMS derivatives. The samples were then stored at -30° until analysis was carried out. GLC analysis was carried out by injection of from 0.5-2.0 ul of the silylation mixture directly onto the column of the gas chromatograph.

RESULTS

Isolation of Permissive Strains. In attempting to isolate strains of *R. sphaeroides* able to produce H_2 from substrates that are non-permissive for H_2 production in the wild-type, initial efforts used N-methyl N'nitro-N-nitrosoguanidine as a mutating agent. Cells were incubated in a 50 mM solution of nitrosoguanidine for 30 min. The cells were collected by centrifugation, washed 4 x in minimal medium to remove the mutagen and plated on minimal agar plates with either an agar overlay to trap evolved H_2 gas or an agar overlay containing 2,3,5-triphenyl 2 H-tetrazolium chloride (TTC). TTC has been used with blue-green bacteria to visualize nitrogenase activity.

Trapping of H_2 gas by agar overlays appeared to fail, since controls of gas-producing cultures did not produce visible pockets of gas. It is possible that agar allows H_2 gas to diffuse out without forming a pocket. It is also possible that the techniques were not sufficiently anaerobic to prevent deactivating the nitrogenase enzyme. Overlays containing TTC were unsuccessful, showing high levels of dye reduction over the entire plate with no specificity. This again may be due more to technique than to the

efficacy of the test. This compound is also used as an indicator of respiration in aerobic systems. As R. sphaeroides can grow marginally under aerobic conditions, the dye can be reduced even with traces of O_2 in the medium.

It was observed that cells grown aerobically on nutrient agar plates had an enhanced ability to produce H_2 when subsequently grown under conditions allowing H_2 production. To isolate these spontaneous mutant strains of R. sphaeroides able to produce H_2 gas from glucose, gluconate or other substrates from the wild-type strain, the wild-type cells were spread on 2% (w/v) agar plates made with rich medium and 10 mM of the particular substrate. These plates were grown aerobically in the light. The small pigmented colonies appearing first were removed and grown anaerobically in rich medium with the appropriate carbon source in the light. This process was repeated twice with the newly isolated cells. Cultures passed three times were used to innoculate minimal media with 10mM carbon source in 25 ml tubes with traps. These tubes were placed in the light at 27°. Cultures corresponding to the tubes producing H were retained. In this way, strains capable of producing H_2 when grown on glucose (Glc$^+$), gluconate (Gnt$^+$) or fructose (Frc$^+$) were isolated.

Metabolism of Substrates. In studies using unlabeled substrates, wild-type R. sphaeroides utilized malate and lactate efficiently, both in terms of maximum rate and in terms of total conversion of substrate to H_2 (Fig. 2-1). With glucose as substrate, only about one culture in ten produced any H_2. Relatively little H_2 was produced and only after a considerable lag period. When gluconate was used as the substrate, no H_2 was produced. Cultures grown on fructose or sucrose produced H_2 readily,

but with only 25-40% efficiency. Citrate or ethanol supported no growth above that of controls on minimal medium alone, and such cultures did no produce H_2. The Glc$^+$ strain produced H_2 when supplied with malate or lactate with the efficiency of the wild-type and produced high levels of H_2 when supplied with glucose (Fig. 2-2). The Gnt$^+$ strain also used malate or lactate with efficiencies similar to the wild-type, and also made appreciable amounts of H_2 when supplied with gluconate. The Frc$^+$ strain utilized fructose to a much higher degree than did the wild-type.

Stoichiometry. Cultures of the wild-type Glc$^+$, Gnt$^+$ and Frc$^+$ strains were assayed quantitatively using system (ii) for substrate conversion using ^{14}C-labeled substrates (Table 2-1). In some cases, the wild-type cells converted glucose to the equivalent amounts of H_2. When this occurred, an average of 24% of the substrate was converted to H_2. $^{14}CO_2$ recovered from the system was somewhat greater, consisting of 34% of the ^{14}C-glucose initially present. The non-CO_2 radioactivity remaining was mostly in starting material (U-^{14}C-glucose), although on paper chromatography a compound with Rf similar to that of gluconate was also observed.

Malate was converted by the wild-type to 57% of the theoretical H_2 and 60% of the theoretical CO_2. Lactate as the carbon source for the wild-type gave 48% conversion to H and 58% of the theoretical CO_2. For these two substrates, although some (<5%) starting material remained, most non-CO_2 radioactivity was found in insoluble carbohydrate polymers.

The Glc$^+$ strain with glucose as substrate yielded an essentially complete (99%) conversion to H_2, with 91% conversion to CO_2 and with no apparent substrate remaining after H_2 production had ceased. It should be noted that the extent of glucose-supported H_2 production of Glc$^+$ was much greater than for the wild-type *R. sphaeroides*.

The Gnt$^+$ strain with gluconate as substrate gave an average of 42% conversion to H_2 and 57% conversion to CO_2. The wild-type strain did not produce H_2 from gluconate.

Frc$^+$ strain yielded 58% of the theoretical H_2 and 68% of the CO_2.

Table 2-2 shows comparable data obtained with the modified Hungate tubes of system (iii). The gas pressure in system (iii), a closed system, continues to increase due to production of H_2 and CO_2 in the time intervals between the samplings. This may account for the lower yields from glucose. However, the results are qualitatively the same as those obtained with system (i), which was maintained at atmospheric pressure. As can be seen, the conversion by Glc$^+$ of glucose to H_2 accounted for 36% of the maximum possible conversion. The wild-type strain converted 9% of the available glucose to equivalent amount of H_2 and CO_2. In both cases, an atmosphere of N_2 decreased the yield of H_2 and reduced the ratio of H_2/CO_2 suggesting that N_2 was serving as an alternative electron acceptor in place of protons. HN_4Cl at a concentration of 5 mM completely suppressed H_2 production in the modified Hungate tubes, along with a lesser inhibition of CO_2 production.

The possible identities of low molecular weight compounds formed during H_2 production were investigated by GLC analysis. Spent culture media of the Glc$^+$ mutant and the wild-type were trimethylsilylated and analyzed by GLC in these experiments. The wild-type organism formed gluconate and an unknown compound from growth on glucose. This unknown migrated in the GLC assay with a retention time nearly identical to the 5-carbon sugars and sugar acids and I have termed this unknown material "C5 sugar acid".

DISCUSSION

It was determined that glucose did not support extensive H_2 production by the wild-type. What H_2 is produced occurs only with a long (48 hr) lag period. Gluconate did not support H_2 production in the wild-type at all. However, selection of the $\underline{Glc}^+$ and $\underline{Gnt}^+$ mutants capable of H_2 production on these substrates was accomplished without difficulty by successive subculturing on a mineral base medium containing glucose or gluconate as the sole carbon source in an aerobic environment. Such "mutants" appear to arise spontaneously to high frequency. These strains were stable when maintained on a non-specific (rich) medium without the appropriate carbon compound. They would produce H_2 when returned to minimal conditions and the carbon source with only the usual lag to deplete the fixed nitrogen in the system. These mutant strains may be altered in their increased capacity to convert glucose to C-3 metabolites using the steps of the Entner-Doudoroff pathway, reported to be the principal degradative pathway for glucose (17,76), or via the Embden-Meyerhof pathway. It is of interest that the wild-type strain excreted significant quantitites of a metabolite migrating with the same solubility (paper chromatography) and retention time (GLC) as gluconate, which could arise from the Entner-Doudoroff or oxidative pentose phosphate pathways. Evidently, the oxidation of glucose to gluconate precedes the phosphorylation step which is required for subsequent reactions. The build-up of glucose carbon in gluconate may indicate that gluconate phosphorylation is a partially rate-limiting step in this metabolic pathway.

It should be noted, since CO_2 is generally found at greater than stoichiometric amounts relative to H_2 (and its production may actually be even higher), that other pathways not leading to complete metabolism of the substrates to H_2 and CO_2 may occur. It may also be that H_2 is being recycled. R. sphaeroides can grow photoautotrophically on H_2 and CO_2 and the uptake stoichiometry would not be expected to be the same as that from H_2 production. Since the Glc^+ strain gave essentially complete stoichiometric conversion, it is clear that reuptake and/or incomplete conversion was minimized. It may be that the direct metabolism of substrate yields CO_2 and reducing potential (such as NADH or NADPH) and that the nitrogenase system is not directly linked to substrate metabolism. H_2 yields might be expected to reflect the excess reducing power not diverted into growth. In such a case, the stoichiometry may not show a complete conversion.

TABLE 2-1. Recovery of H_2 and CO_2 from Gas-Producing *R. sphaeroides*

All cultures were grown anaerobically on minimal medium with 0.2% yeast extract and 10 mM carbon substrate (pH 7.2) in the light. Percent values in parentheses are of theoretical maximum yields assuming complete stoichiometric conversion. All values, average of four trials. W.T.: wild-type *R. sphaeroides*; *Glc*$^+$: gluconate-utilizing mutant; *Gnt*$^+$: gluconate-utilizing mutant.

Cell type	Substrate	H_2 gas evolved	CO_2 gas eluted	Label recovered
W.T.	Glucose, 0.5[a]	1.4 (24)[b]	1.0 (34)[b]	(91)[b]
W. T.	Malate, 0.5	1.7 (57)	1.25 (60)	(92)
W. T.	Lactate, 0.5	2.9 (48)	1.7 (60)	(91)
Glc$^+$	Glucose, 1.0	12.0 (99)	5.45 (91)	(95)
Gnt$^+$	Gluconate, 0.5	2.3 (42)	1.7 (57)	(90)

(a) Initial amount of substrate in millimoles in each culture.

(b) Values indicate, in millimoles, recovery of material after cessation of gas evolution.

TABLE 2-2. H_2 Yields from Glucose, Wild-type and Glc$^+$ Strain

Cells were grown on minimal medium with the appropriate nitrogen source and 20mM glucose in the light.

Strain	Atmosphere	N source	H_2 evolved	CO_2 eluted	Ratio	Conversion
Glc^{+a}	Ar	0.5% YE[b]	436[c]	197[c]	2.2	36%[d]
Glc$^+$	N_2	"	167	128	1.3	14%
W.T.	Ar	"	99	98	1.0	9%
W.T.	N_2	"	33	32	1.0	3%
Glc$^+$	N_2	5 mM NH_4^+	0	117	0	0
W.T.	Ar	"	0	34	0	0

(a) Glc+: glucose-utilizing mutant; W.T. : wild-type strain.

(b) YE: yeast extract.

(c) Values for H_2 and CO_2 are the average of three replicate samples and are expressed in terms of umole recovered per mg dry weight of cells.

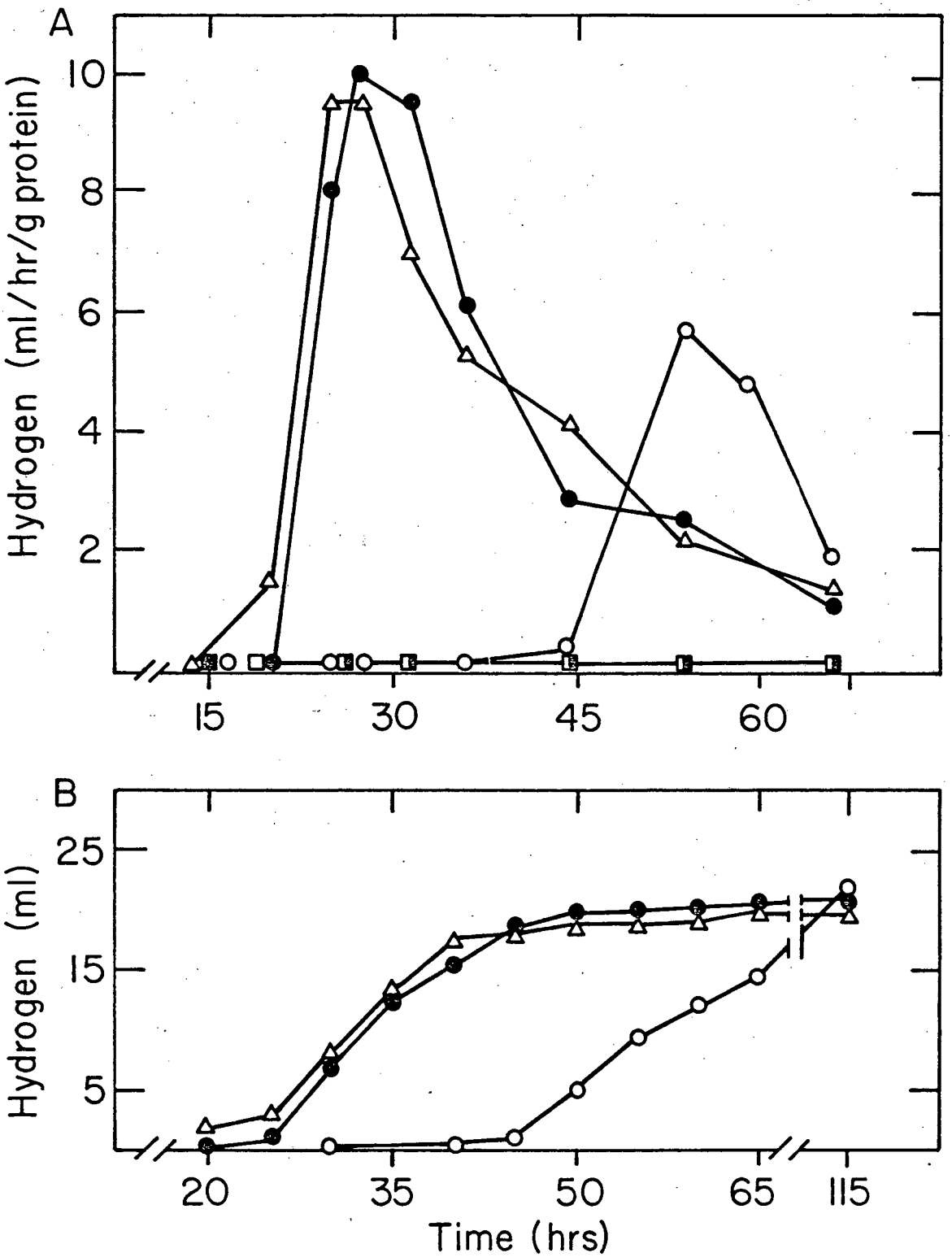
(d) Percent conversion of glucose to H_2 based on the maximum theoretical yield.

FIGURE LEGENDS

2-1 H_2 evolution by wild-type R. sphaeroides. Wild-type cultures were grown in minimal medium with 10 mM of the indicated carbon source anaerobically at 8,000 lux illumination. Symbols: O-O , glucose; Δ-Δ, lactate; ●-● , malate; □-□, gluconate; ■-■, citrate. (A) Rate of H_2 evolution vs. time. (B) Total H_2 evolved vs. time.

2-2 H_2 evolution by Glc^+ and Gnt^+ mutants of R. sphaeroides. The appropriate strains were grown anaerobically in minimal medium and 10 mM of the indicated carbon source at 8,000 lux illumination. Symbols: O-O, glucose, Glc^+ strain; ●-●, malate, Glc^+ strain; □-□, gluconate, Gnt^+ strain; ■-■, malate, Gnt^+ strain. (A) Rate of H_2 evolution vs. time. (B) Total H_2 evolved vs. time.

2-3 GLC analysis of TMS derivatives of culture media. After H_2 production, cultures were freeze-dried, trimethylsilylated and separated by GLC, using a flame ionization detector (FID). Symbols: α-glc, TMS derivative of α-glucose; β- glc, TMS derivative of β-glucose; gnt, TMS derivative of gluconic acid. (A) Glc^+ strain. (B) Wild-type strain.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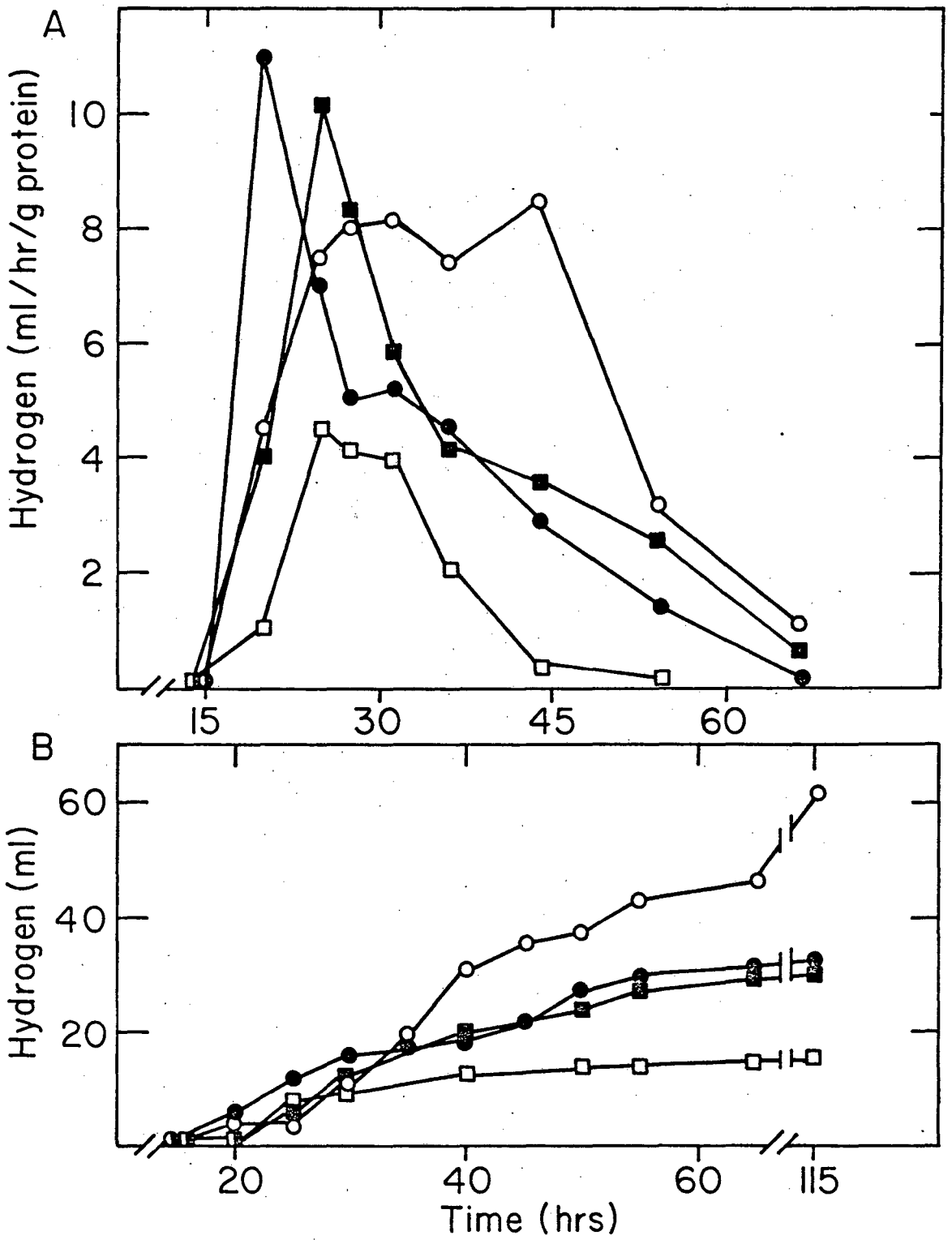
Hydrogen (ml/hr/g protein)
10
8
6
4
2
15
30
45
60
B
Hydrogen (ml)
25
15
5
20
35
50
65
115
Time (hrs)

XBL789-4222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Hydrogen (ml/hr/g protein)
10
8
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2
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30
45
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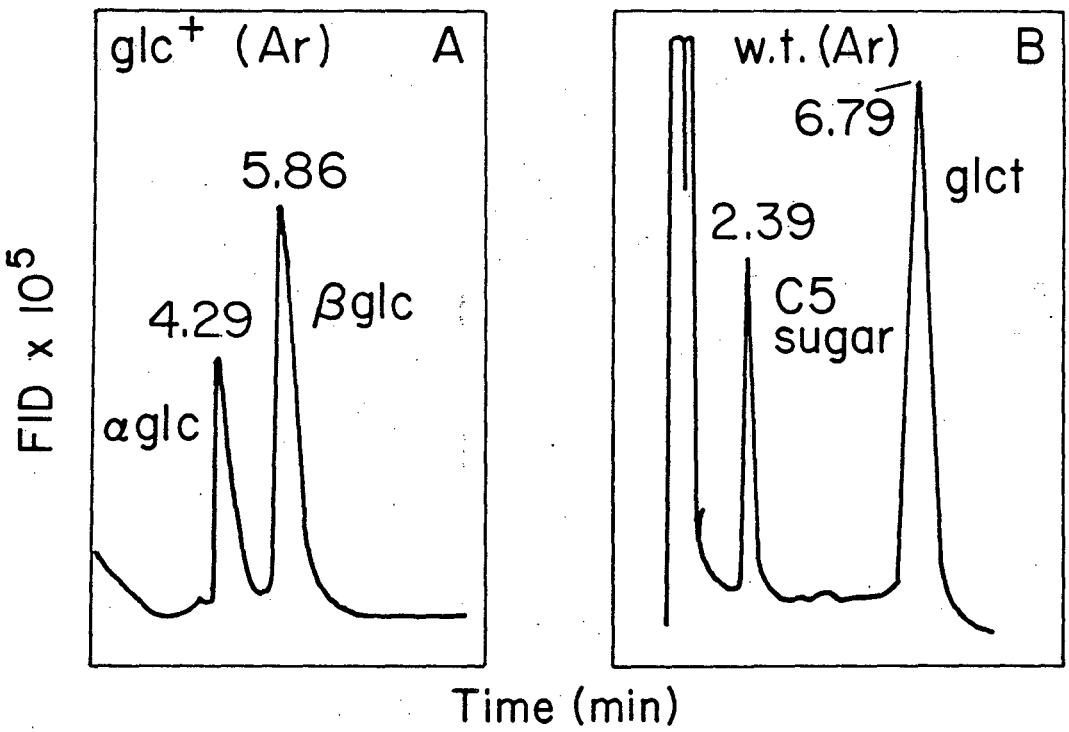
B
Hydrogen (ml)
60
40
20
20
40
60
115
Time (hrs)

XBL789-4221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A
5.86
4.29
βglc
αglc
FID x 10^5
w.t. (Ar)
B
6.79
glct
2.39
C5
sugar
Time (min)

XBL 788-4141

CHAPTER 3. ENERGY CHARGE

INTRODUCTION

After I determined that R. sphaeroides utilized a nitrogenase enzyme to produce H_2 and that H_2 production occurred at high rates only when the cultures were incubated in the light, I became interested in determining the energy charge of the organism under conditions both allowing H_2 production, and when H_2 production was suppressed. Energy charge in this sense refers to the ratio of high-energy phosphate bonds in ATP and ADP to the total adenylate pool, as formulated by Atkinson (6,7):

$$\frac{1}{2}\left(\frac{2[ATP] + [ADP]}{[ATP] + [ADP] + [AMP]}\right) = \text{energy charge}$$

Enzymatic production of H_2 by nitrogenase uses a minimum of four ATP molecules for each molecule of H_2 generated, or two ATP molecules per electron transferred (21,82). This could be a heavy drain on cells that are rapidly converting a reduced compound to H_2. Generally, such ATP requiring processes are associated with an energy charge of 0.8 or greater in the cell. Organisms that utilize an oxidative electron transport chain to reduce molecular oxygen can generate up to three ATP per electron. Since R. sphaeroides produces H_2 anaerobically, it was unclear if oxidative metabolism could supply sufficient ATP for nitrogenase action. In the light, this organism can generate ATP via cyclic photophosphorylation and this may be the source of energy for H_2 production. In either case, the rapid utilization of ATP in cells maximally producing H_2 could yield a relatively low average energy charge.

In vitro studies of nitrogenase suggest that this enzyme is provided with electrons by reduced ferredoxin. If metabolism of the substrate is through typical known systems such as glycolysis, the tricarboxylic acid cycle or the oxidative pentose phosphate pathway, then electrons probably would appear as NADH or NADPH. The midpoint potentials for the pryidine nucleotide and ferredoxin oxidation/reduction reactions, assuming equal amounts of oxidized and reduced species, are -0.31 and -0.43 respectively. Thus, if reduction of ferredoxin by NADH or NADPH were to occur in a reverse ferredoxin-NADP oxidoreductase reaction $2Fd_{OX} + NADH \rightleftharpoons 2Fd_{RED} + NAD^+$, then the ratio of reduced to oxidized pyridine nucleotide must be high. In this section I present data on pyridine nucleotide levels and energy charge.

MATERIALS AND METHODS

Organism and Culturing. Wild-type and Glc^+ mutant *R. sphaeroides* were maintained and cultured under conditions as described in Chapter One.

Isotachophoresis Experiments. Cultures of *R. sphaeroides* were grown in Hungate-type tubes in minimal medium containing 5 mM glutamate and 10 mM malate or fructose. The atmosphere was Ar. Cultures were kept in the light (1000 lux) throughout the experiment. The onset of H_2 gas production was determined by either gas-liquid chromatographic analysis of the culture atmosphere (described in Chapter Two) or by flame test for combustion of a sample drawn from the culture atmosphere.

When gas production appeared maximal, half of the cultures received NH_4Cl to produce an 8 mM solution. After a further incubation of one

hour, samples were taken into 80% methanol and NaOH, pH 8, or HCl, pH 3.5. Cultures were then frozen. This procedure to maintain the oxidized and reduced forms of the pyridine nucleotides was from Lendzian and Bassham (52a).

Analysis of cell material was isotachophoresis. 10-20 µl of the samples were injected into an LKB model 2127 Tachophor unit with a double loop capillary. Leading buffer was 0.5% hydroxypropyl methyl cellulose (HPMC) in 5 mM HCl, pH 3.9. Terminal buffer was 5 mM hexanoic acid. Columns were run at constant current in decreasing steps, from 250 A to 50 A. The column was maintained at 20°. Detection of peaks was by ultraviolet absorbance. Peaks were measured with a modified Hewlett-Packard automatic integrator. The column was calibrated with known amounts of standard material.

Labeling Experiments. Cells of the wild-type *R. sphaeroides* and the Glc^{+} mutant capable of producing H_2 from glucose were grown as described in Chapter One on rich medium. Cultures in the log phase of growth were harvested by centrifugation, washed two times in minimal buffer and used to inoculate (10% v:v) Hungate-type tubes containing minimal medium, 5 mM glutamate and either 20% mM glucose or malate. The atmospheres were flushed with Ar and the cultures grown in the light at 27°. At the onset of H_2 production (10-15 hrs), all tubes received $^{32}P\text{-}PO_4$. Samples were taken at intervals following the addition of tracer and killed in 80% methanol. This material was assayed by two-dimensional paper chromatography, autoradiography and Geiger-counting as described previously (44,66). Adenylate spots were identified by ultraviolet fluorescence of known compounds applied with the sample to the paper.

RESULTS

Isotachophoresis. Cells grown with malate as a substrate showed about three times as much NAD^+ as $NADP^+$ regardless of whether they are producing H_2. For cultures growing with fructose as carbon source, the data were similar. However, the amount of NAD^+ was higher and that of $NADP^+$ was lower when the cells were producing H_2 than when they were inhibited with NH_4Cl. NADPH was not detectable in cultures grown on fructose.

Cells grown with malate as a substrate had about the same levels of total adenylate, regardles of the extent of gas evolution. Cultures on fructose had similar levels of adenylates to those during H_2 production. The addition of NH_4Cl led to a fourfold increase in AMP. Ratios of $NADH/NAD^+$ and $NADPH/NADP^+$ and cellular energy charge are presented in Table 3-1.

^{32}P-phosphate Labeling. All culture of Glc^+ strain grown on glucose or malate and all wild-type cultures grown on malate produced H_2 regardless of whether CO_2 was present in the atmosphere. Culture of the wild-type grown on glucose did not produce H_2 and were considered to be stationary in growth. Their cellular amounts and energy charge are shown in Table 3-2. Note that all cultures, whether fixing CO_2, producing H_2 or in stationary growth, seem to maintain an energy charge around 0.5.

ATP, ADP and AMP were identifiable on paper chromatograms. The pyridine nucleotides were not found to be significantly labelled. It is possible that these compounds were not stable under conditions of development of paper chromatograms and were lost.

DISCUSSION

The data on *in vivo* levels of adenylates and the cellular energy charge in *R. sphaeroides* from both isotachophoretic analysis and radioactive phosphorous labeling of cellular material suggests that the energy charge is about 0.5 for this organism grown photoheterotrophically regardless of whether it is producing H_2, fixing CO_2 or in stationary growth. Since enzymatic H_2 production requires large amounts of ATP, and H_2 production in certain cultures was at high rates, it would be consistent that ATP was turning over rapidly.

Atkinson holds that an energy charge around 0.8 is the cross-over point for activation of ATP-producing reactions and ATP-utilizing reactions. As the energy charge decreases, ATP synthesis is stimulated and energy requiring reactions are inhibited. This would mean that a steady-state energy charge of 0.5, as in *R. sphaeroides*, would be stimulating generation of ATP.

The regulation of energy production by energy charge would suggest that *R. sphaeroides* culture in stationary growth, unable to produce H_2 or fix CO_2, should show a high energy charge, particularly since cyclic photophosphorylation could continue to occur. This was not seen. The energy charge was the same for all cultures. It was also observed that $NADH/NAD^+$ and $NADPH/NADP^+$ ratios were about 0.2. If the production of H_2 was driven by reducing power in pyridine nucleotides, then the relevant half-reactions would be

$$\begin{array}{ll} NADH + H^+ \rightleftharpoons NAD^+ + 2e^- & E'_o = 0.32 \\ 2H^+ + 2e^- \rightleftharpoons H_2 & E'_o = -0.41 \\ \hline NADH + H^+ \longrightarrow NAD^+ + H_2 & E'_o = 0.09 \end{array}$$

This reaction would be pH dependent. The Nernst equation reads

$$\Delta G'_o = \eta Fe'_o = -RT \ln [H_2] [NAD^+]/[H^+] [NADH]$$

Since G'_o is defined at pH 7, $[H^+] = 1$. With the addition of the observed $[NAD^+]/[NADH]$ ratio of 5, then

$$\Delta G'_o = -\eta Fe'_o = -RT \ln (5[H_2])$$

$n = 2$ $F = 23066$, $\log = 2.303 \ln$, $R = 1.986$, $T = 310°$ K

$$G'_o = -(2)(23066)(-0.09v) = 4152 \text{ cal}$$
$$= (-1.986)(310)(2.303) \log 5[H_2]$$
$$= -1418 (\log 5 + \log [H_2]) = -1418 \log [H_2] - 991$$

Therefore $5143 = -1418 \log [H_2]$, $\log [H_2] = -3.63$

So $[H_2] = 2.4 \times 10^{-4}$ atmosphere

This would suggest that H_2 production might be possible to a limited extent driven by reduced pyridine nucleotides. However, actual production is several orders of magnitude higher. It was observed in closed systems that $[H_2]$ approached 1 atmosphere.

Measurements of the pyridine nucleotides were from broken cell preparations and might not reflect concentrations in localized regions. If a localized $[NADH]/[NAD^+]$ ratio was on the order of 10^3, then $[H_2] = 1.2$ atm and these reactions could account for the observed H_2 production However, the evidence of a minimal contribution of hydrogenase versus nitrogenase suggests that an ATP driven reaction is what is actually occurring.

It is clear that the energy charge and reducing potential of R. sphaeroides are not measurably high, even though the production of H_2, which requires energy and reducing power, proceeds at good rates. It is possible that an active nitrogenase provides sufficient pull on the metabolic pathways that ATP and NADH do not accumulate throughout the cell. It is also possible that electrons are transferred in some way other than through reduced pyridine nucleotides.

The oxidative pentose phosphate pathway yields NADPH as its sink for electrons, as does the Entner-Doudoroff pathway. Glycolysis and the tricarboxylic (TCA) cycle produce NADH. The fact that the level of total NADP is only about one-third that of NAD suggests that glycolysis and the TCA cycle may be more significant as the pathways for substrate metabolism.

A larger view of R. sphaeroides shows us an organism that has an excess of light energy and an excess of reduced carbon, but is unable to grow due to limited fixed nitrogen. This organism is able to regulate itself such that its energy charge and reducing potential do not reach extremes. It appears to do this by producing H_2.

TABLE 3-1. Energy Charge and Reducing Potential Determined by Isotachophoresis for *R. sphaeroides*

Wild-type cells were grown anaerobically on minimal medium with 10 mM of the appropriate carbon substrate in the light. Samples were killed in 80% methanol and NaOH, pH 8 or 80% methanol and HCl, pH 3.5 and then frozen. Material was assayed by isotachophoresis with conditions as in text.

Substrate	H_2 Production[a]	Energy Charge[b]	$NADH/NAD^+$	$NADPH/NADP^+$
malate	+	0.48	0.21	0.16
malate	-	0.54	0.19	0.18
fructose	+	0.37	0.19	--
fructose	-	0.18	0.28	--

(a) "+" indicates H_2 production; "-" indicates inhibition of H_2 production with 8 mM NH_4Cl.

(b) Energy charge as described by Atkinson (6,7).

TABLE 3-2. Energy Charge and Adenylate Labeling from ^{32}P-Phosphate with *R. sphaeroides*

Cells of the wild-type or *Glc*$^+$ strain of *R. sphaeroides* were grown in minimal medium with 20 mM of the appropriate carbon substrate until H_2 production was generally observed. ^{32}P-phosphate (1 mM) was added and samples killed and assayed as described in the text. Inhibitor or bicarbonate were added where indicated one hr before the radioactive label.

Strain	Substrate	Bicarbonate[a]	Inhibitor[b]	ATP	ADP	AMP	Energy Charge[c]
W.T.	malate	-	-	.008	.021	.008	0.52
W.T.	"	-	+	.008	.028	.009	0.44
W.T.	"	+	-	.043	.043	.02	0.62
W.T.	"	+	+	.010	.016	.007	0.56
W.T.	glucose	-	-	.004	.005	0	0.72
W.T.	"	-	+	--	--	--	--
W.T.	"	+	-	.012	.006	.003	0.71
W.T.	"	+	+	.006	.007	0	0.73
Glc$^+$	malate	-	-	.007	.011	.003	0.59
Glc$^+$	"	-	+	.007	.008	0	0.77
Glc$^+$	"	+	-	.008	.014	.007	0.52
Glc$^+$	"	+	+	.008	.007	0	0.75
Glc$^+$	glucose	-	-	.030	.043	.024	0.53
Glc$^+$	"	-	+	.024	.031	.011	0.60
Glc$^+$	"	+	-	.025	.041	.043	0.42
Glc$^+$	"	+	+	.029	.044	.024	0.53

(a) 10 mM $NaHCO_3$ was added where indicated and the CO_2 traps were not used.

All other cultures had solid NaOH in side-arm traps to remove CO_2.

(b) 5 mM fluoroacetate was added where indicated to suppress tricarboxylic acid cycle activity.

(c) Energy charge was as described by Atkinson (6,7).

CHAPTER 4. RADIOTRACER STUDIES OF CARBON AND PHOSPHATE FLOW

INTRODUCTION

Having determined that R. sphaeroides was suitable for large-scale H_2 production by determining the factors necessary to maximize this process and isolating highly efficient strains that could utilize readily available substrates, it became important to determine the metabolic pathways actually used by the cell when making H_2. From existing knowledge of metabolism in general and specific pathways of this organism in particular, combined with data on intracellular energy levels and reducing potential and the absolute levels of intermediates, one could then locate possible sites of regulation. This would suggest ways of modifying the system so as to potentially further increase efficiency and/or yields.

The literature has suggested specific pathways in substrate metabolism and in carbon fixation, although there remain uncertainties. Almost all of the Rhodospirillacae have been examined and they are all different. They also behave differently when the conditions of culturing change.

Szymona and Doudoroff (76) reported that the wild-type strain of R. sphaeroides could not utilize gluconate and grew poorly with glucose, fructose or mannose as growth substrates. These cultures accumulated aldonic acids and 2-keto-3-deoxygluconate. Mutants capable of growing well on glucose without acid accumulation acquired the enzyme "phosphogluconic acid dehydrase" and oxidized hexoses via the Entner-Doudoroff pathway. They claimed that the Embden-Meyerhof pathway had limited function due to low aldolase activity and that the oxidative pentose

phosphate pathway was not fuctional due to low or absent phosphogluconic acid dehydrogenase activity.

Conrad and Schlegel (17) reported that R. sphaeroides metabolized glucose photoheterotrophically via the Entner-Doudoroff pathway, while fructose was metabolized predominantly via the Embden-Meyerhof pathway.

Anderson and Fuller (2,3a,b), working with R. rubrum, reported that photoheterotrophically grown cells with malate as substrate had very low levels of ribulose 1,5 bisphosphate (RuBP) carboxylase activity and that the reductive pentose phosphate (Calvin) cycle operated at significant levels only under strict autotrophic conditions. They demonstrated the presence of the tricarboxylic acid (TCA) cycle and enolase enzymes in heterotrophically-grown cells, although α-ketoglutaric dehydrogenase levels were low. In other work, they showed that photoheterotrophically-grown R. rubrum fixes ^{14}C-$NaHCO_3$ initially into glycolate (2). The label rapidly moved to malate, phosphoenolpyruvate and TCA cycle intermediates. They reported that this fixation was relatively low in providing carbon skeletons compared to the direct utilization of the growth substrate malate.

Lascelles (52) on the other hand, reported that photoheterotrophically-grown R. sphaeroides had significant RuBP carboxylase activity. Kornberg and Lascelles (49) reported that TCA cycle R. sphaeroides to be sufficient to provide both energy and carbon skeletons for growth. This was supported by similar work by Eley, et al. (23). They also found low levels of isocitratase, suggesting that the glyoxylate cycle was low in R. sphaeroides.

In reviewing this literature, it was clear that no one had ever looked at the metabolism of R. sphaeroides in vivo using radioactive sub-

strates and isolating the labeled intermediates. The majority of the reports measured specific enzymes in soluble cell fractions. Some workers used ^{14}C-$NaHCO_3$ to determine gross fixation, or used labeled substrates to look at released $^{14}CO_2$. My interest was to identify qualitatively and quantitatively the products of metabolism and determine patterns of labeling in cells producing H_2.

Since CO_2 is released in stoichiometric amounts with any substrate producing H_2, it was important also to look at the fixation of CO_2 in the highly reducing atmosphere produced. If this was significant, the efficiency of the system for producing H_2 would be decreased. In this case, it would be useful to be able to minimize CO_2 fixation.

MATERIALS AND METHODS

Radiotracer Labeling Experiments. Cells of the wild-type, glucose-utilizing (Glc$^+$) and fructose-utilizing (Frc$^+$) strains were grown and collected as described earlier and used to innoculate (10% v/v) serum-capped 15 ml side-armed tubes (Fig. 4-1) containing minimal medium, 5 mM glutamate and 20 mM carbon source. The atmospheres were flushed with argon and the side-arms had solid NaOH as CO_2 traps. Cultures were grown in the light. When H_2 production had generally begun, the appropriate radiotracers were added. Samples were killed in 80% methanol. These were applied to 4 cm Dowex 50, 200-400 mesh columns in Pasteur pipettes. These were eluted sequentially with 3 x 1 ml water, 3 x 1 ml 1N pyridine and 3 x 1 ml 3N NH_4OH. Small amounts of each fraction were assayed for radioactivity by scintillation counting. The aqueous fraction was reduced in volume under a N_2 gas stream. Pyridine and ammonia fractions were taken

to dryness under N_2. All fractions were resuspended in minimal amounts of 80% methanol, spotted on paper and chromatographed as described previously (44). Chromatograms were assayed by autoradiography and scintillation counting as described previously (66).

Unknown radioactive areas on the chromatograms were eluted with water and layered onto 4 cm acetic acid-charged Dowex 1 columns in Pasteur pipettes. These were eluted sequentially with 3 x 1 ml water, 3 x 1 ml 2N acetic acid, 3 x 1 ml 2N formic acid and 3 x 1 ml 2N HCl. Small amounts of each fraction were counted for radioactivity by scintillation. Fractions were reduced in volume under N_2, spotted on paper, chromatographed and analyzed as above.

Eluted unknown material was treated with concentrated HCl in Warburg flasks containing Protosol (New England Nuclear) in the center well. After two hrs reaction, the center well material was counted by scintillation and the remaining material was paper-chromatographed as described above.

Dinitrophenyl hydrazone derivatives were prepared using standard techniques. Amino acids on paper chromatograms were visualized by ninhydrin reaction.

Material on paper chromatograms associated with the origins was hydrolyzed in 2N HCl in sealed ampules heated to 140° for one hour. The hydrolyzates were chromatographed and analyzed as above.

RESULTS

^{14}C-substrate and ^{32}P-phosphate metabolism. Initial experiments with ^{14}C-labeled substrates did not lead to acceptable levels of labeling of metabolic intermediates, although information on stoichiometry and primary end products was accumulated. In these experiments, minimum volumes were 12-15 ml and the specific activities of the labeled compounds were kept low. Volumes of this size were necessary to ensure measurable amounts of H_2 production. Later experiments were in 5 ml of medium and higher specific activities and yielded reproducible labeling of intermediate compounds. This was possible from improvement in my anaerobic techniques which preserved H_2 producing activity.

The primary carbon-containing end product in cultures producing H_2 was CO_2, which appeared in stoichiometric amounts. No other compound accumulated in a stoichiometric fashion when H_2 was being produced. Intermediates were commonly observed in the μgm per gm of dry cell weight range (Table 4-1).

In order to test the activity and contribution of the tricarboxylic acid cycle to substrate metabolism, inhibitors of aconitase were added to cultures under conditions allowing H_2 production. Monofluorocitrate was initially tested and no inhibition of H_2 production was observed at concentrations to 20 mM. Monofluoroacetate, however, decreased H_2 production up to 75% at concentrations of 10 mM (Table 4-2). This suggests that monofluorocitrate may not have been taken up by the cells. Higher concentrations of fluoroacetate did not appear to yield further inhibition. The wild-type strain was inhibited 75% by 10 mM fluoroacetate when malate was

the substrate. Inhibition was 30% when fructose was the substrate and 60% when lactate was the substrate. With the Glc$^+$ strain, a 60% decrease in H_2 production was observed from fluoroacetate when malate was the substrate. Similarly, cells grown on glucose had a 60% inhibition of gassing and cells with fructose as substrate decreased 30%.

The Frc$^+$ strain growing on fructose had a 60% decrease in H_2 yield in the presence of 10 mM fluoroacetate.

A series of experiments was undertaken to characterize the wild-type metabolism of R. sphaeroides growing on malate or glucose under conditions allowing H_2 production. Since the wild-type seldom produced H_2 when grown this way on glucose, parallel cultures of cells with malate or glucose were used. When H_2 production was obvious in the cultures grown on malate, the appropriate radioactive tracers were added to both malate and glucose cultures. In cases where fluoracetate was added to inhibit the TCA cycle, the addition was made 30 to 60 min before the addition of the tracers.

When cells of R. sphaeroides grown anaerobically in the light with ^{14}C-glucose were killed in 80% methanol and chromatographed on paper in water-saturated phenol (pH 4.3) and butanol: propionic acid, radioactivity appeared in areas that co-chromatogrpahed with aspartic and glutamic acids. However, when this material was passed through Dowex 50 to isolate amino acids, the pyridine fraction containing neutral and acidic amino acids showed, after paper chromatography and autoradiography, no radioativity in the aspartate area and only small amounts in the glutamate area. The water wash fraction containing sugars and carboxylic acids retained the radioactivity associated with those two compounds. Column

chromatography of these two unknown radioactive spots on Dowex 1 to separate them by pK value showed the spot associated with aspartate eluting with HCl and the spot associated with glutamate eluting with formic acid (Table 4-3).

When cells grown anaerobically in the light with ^{32}P-phosphate were killed and chromatographed as above, no ^{32}P-label appeared in areas co-chromatographing with aspartate or glutamate.

It was observed that both unknowns produced gas when treated with acid. The unknowns were treated with concentrated HCl in Warburg flasks and the released CO_2 collected in Protosol. It was found that the spot associated with glutamate released 4% of its radioactivity as $^{14}CO_2$ and the spot associated with aspartate released 9% of its radioactivity as $^{14}CO_2$. Chromatography in the standard solvents showed the decarboxylation product of the aspartate-associated unknown to have an R_f value of 50% that of aspartate in the phenol direction and 60% of the aspartate R_f value in butanol: propionic acid. The decarboxylation product of the glutamate-associated unknown did not remain on chromatograms.

Dinitrophenol hydrazones of the two unknowns were prepared. Only the material associated with glutamate formed a DNP derivative.

It was observed that ^{14}C-gluconic acid appeared to co-chromatograph with aspartate in water-saturated phenol (pH 4.3) and in butanol: propionic acid. It eluted from a Dowex 1 column with HCl.

The aspartate-associated unknown will be referred to as gluconate. The glutamate-associated unknown will be referred to as "C5 acid".

<u>Labeling Patterns of the Wild-type strain, Malate Substrate</u>. The wild-type strain grown on malate rapidly metabolized this substrate. All

label in the substrate was gone in 8-10 hrs. Label in intermediates was low (Figs. 4-2 to 4-11). Major ^{14}C-labeled pools from ^{14}C-malate were gluconate and insoluble material, principally polyglucose. These became more heavily labeled with time. There was an initial rise in labeled glucose and fructose that rapidly disappeared. Label in TCA cycle intermediates was low to absent. When ^{32}P-phosphate was added as tracer, labeling appeared and accumulated in fructose-6-phosphate, glucose-6-phosphate and 6-phosphogluconate as well as in insoluble material.

The addition of fluoroacetate to cultures producing H_2 from ^{14}C-malate led to rapid labeling of glucose, fructose, phosphoglycerate, phosphoenolpyruvate, citrate and insoluble material. Labeled pools for glucose, fructose, phosphoglycerate and phosphoenolpyruvate declined over time. Label in citrate remained steady over time, while label in insoluble material, gluconate and C_5 acid increased. In these experiments, fluoroacetate inhibited H_2 production by 50% and the ^{14}C-labeled pool sizes increased tenfold. 90% of the ^{32}P-labeled material was found in insolubles when cultures contained fluoroacetate. Label in 6-phosphogluconate doubled, while that in glucose-6-phosphate and fructose-6-phosphate decreased fivefold. Labeling in the adenylates was unchanged.

The addition of 5 mM NH_4Cl to these cultures, which stopped H_2 production, led to increased levels of glutamate, protein and polyglucose. Subsrate disappeared within 2-3 hrs.

Labeling Patterns of the Wild-type Strain, Glucose Substrate. The wild-type strain utilized glucose relatively poorly, with only 12-15% of ^{14}C-glucose used in 24 hrs. No H_2 was produced. This substrate gave rise within 30 min to fructose, phosphoglycerate, α-keto glutarate and

6-phosphogluconate. The fructose and α-keto glutarate pools then rapidly declined. Gluconate and C5 acid appeared early and rose over several hours to become the dominant ^{14}C-labeled pools. The insoluble material, primarily polyglucose and polyphosphate, increased substantially over time. Label in glycolate and fructose-6-phosphate appeared later in low amounts. The TCA cycle intermediates were unlabeled.

Fluoroacetate addition appeared to generally inhibit metabolism of glucose. ^{14}C-label appeared in polyglcose, gluconate, C5 acid, citrate, malate and 6-phosphogluconate and increased over time. Glycolate, fructose and α-keto glutrarate rose immediately, then slowly declined over time. Glycolate rose quickly and maintained a steady level of labeling.

In one experiment, cultures were grown on malate until H_2 production was observed, then ^{14}C-glucose was added. The tracer was completely metabolized within 8 hrs. Less than 10% of the radioactivity was found in $^{14}CO_2$ 60% of the radioactivity appeared in gluconate, 13% in C5 acid and 11% in polyglucose. Other compounds with ^{14}C-label included glycolate, α-keto glutarate, 6-phosphogluconate and the hexose monophosphates. These pools contained less than 1% each of the starting amount of tracer. The addition of fluoroacetate led to labeling of citrate and to lesser degrees, phosphoenolpyruvate and fructose. Other labeled pools did not appear to be affected by this treatment.

Labeling Patterns of Glc^+ Strain, Malate Substrate. A parallel series of experiments were undertaken to characterize the metabolism of the Glc^+ strain of *R. sphaeroides*. As with the wild-type, cultures were started with unlabeled substrate and grown anaerobically in the light until H_2 production was observed. Inhibitors when appropriate were added,

followed after a period of incubation by the appropriate ^{14}C-labeled substrate or ^{32}P-phosphate. Samples were taken at intervals, killed and analyzed as described above. Data appear in Figs. 4-2 to 4-11.

^{14}C-malate led to label in glycolate and polyglucose early in culture. Glucose, fructose and citrate labeling increased markedly, then declined well before depletion of substrate. Label in polyglucose accumulated to a high level until the depletion of substrate, then declined. Gluconate and C5 acid had no significant labeling. When ^{32}P-phosphate was used as a tracer, glucose-6-phosphate and at lower amounts fructose-6-phosphate and fructose 1,6 diphosphate accumulated label over time. 6-phosphogluconate had a low, steady level of label.

Growth on ^{14}C-malate with the addition of fluoroacetate led to a rapid labeling of glucose, citrate and glycolate. Fumarate, phosphoenolpyruvate, fructose and polyglucose showed label later in the experiments. Glycolate decreased along with substrate depletion. With ^{32}P-phosphate labeling, the hexose monophosphates were at lower levels relative to cultures without fluoroacetate. Fructose 1,6 diphosphate was about the same size. Gluconate and C5 acid remained unlabeled.

Both cultures with and without fluoroacetate produced H_2. Fluoroacetate yielded a 50% inhibition of H_2 production in these experiments. While control cultures without fluoroacetate accumulated the preponderance of non-$^{14}CO_2$ ^{14}C-label as polyglucose, cultures with fluoroacetate showed large pools of citrate and glucose while substrate remained. More ^{32}P-label appeared in polyphosphate in cultures with fluoroacetate.

<u>Labeling Patterns of the Glc$^+$ Strain, Glucose Substrate</u>. The <u>Glc$^+$</u> strain rapidly metabolized ^{14}C-glucose to immediately label fructose,

fructose-6-phosphate, phosphoglycerate and glycolate. The TCA cycle compounds α-ketoglutarate, citrate, malate and fumarate began to be labeled early. α-ketoglutarate and fumarate pools increased several-fold over time. Fructose and phosphoglycerate declined in label with time. 6-phosphoglyconate, fructose-6-phosphate, glucose-6-phosphate and fructose 1,6 diphosphate were labeled by ^{32}P-phosphate in about equal amounts and accumulated over time. Polyphosphate accumulated to a high level and accounted for 80% of the ^{32}P-labeled products. ^{14}C-label appeared in gluconate, C5 acid and polyglucose and increased over time.

Addition of fluoroacetate to these cultures led to rapid and sharp increases in citrate, fumerate, malate, phosphoenolpyruvate and α-keto-glutarate, which all increased with time. 6-phosphogluconate increased in ^{32}P-label relative to the control cultures and glucose-6-phosphate disappeared. Label in all other pools remained essentially unchanged.

The Glc$^+$ mutant used glucose to a significantly greater amount than did the wild-type, with about 30-40% of the labeled substrate utilized after 24 hrs. The wild-type accumulated label in insolubles, gluconate, C5 acid and 6-phosphogluconate. The Glc$^+$ mutant moved more label into polyglucose and much less into gluconate and C5 acid. 6-phosphogluconate was not the dominant monophosphate with the Glc$^+$ mutant.

The addition of ammonium to Glc$^+$ cultures growing on glucose stimulated metabolism of the substrate. A fivefold increase in glycolate and glutamate was observed. Label in insoluble material increased tenfold. Hydrolysis of this material yielded about equal parts glucose and amino acids. Ammonium ion addition led to a decrease in labeled TCA cycle

intermediates. The levels of glyconate labeling were similar to cultures growing on glucose alone.

In one series of experiments, Glc$^+$ cultures were grown on glucose as substrate until H_2 evolution began, then ^{14}C-fructose was added in tracer amounts. Under these circumstances, the tracer disappeared in a few hours. ^{14}C-label in polyglucose, gluconate and C5 acid reached peak levels after 8-12 hrs, then began to decline. The addition of fluoroacetate led to increased levels of labeling for citrate (2X), malate (20X) and fumarate (25X), as well as for glycolate (4X). Polyglucose labeling was little changed. The addition of ammonium ion to cultures producing H_2 resulted in a doubling of ^{14}C-label in insolubles and an inhibition of labeling of gluconate.

When the Glc$^+$ was cultured on ^{14}C-labeled fructose, label appeared in polyglucose, gluconate, glycolate and C5 acid predominantly. Some label was observed in the TCA cycle compounds. Addition of fluoroacetate led to 10-25-fold increases in citrate, malate and fumarate, a fourfold decrease in labeling of polyglucose and a doubling of label in gluconate.

Labeling Patterns of the Frc+ Strain, Fructose Substrate. When Frc$^+$ strain cultures were grown on ^{14}C-fructose, label rapidly appeared in the insoluble material (polyglucose), gluconate, C5 acid, malate, fumarate, citrate and 6-phosphogluconate. No label was observed in hexose monophosphates, α-ketoglutarate or phosphoenolpyruvate. The majority of label appeared in polyglucose and in gluconate.

The addition of fluoroacetate led to a peak of pool labeling at about 8 hrs of incubation, followed by a decline in labeled pool sizes for the remainder of the experiments. Citrate and other TCA cycle compounds were

only lightly labeled in the presence of fluoroacetate. Fluoroacetate inhibited substrate utilization and H_2 production by 80%.

The addition of ammonium ion to cultures on fructose stimulated metabolism of the substrate by 50%. Label in glutamate and in insoluble material increased substantially. Label in the TCA cycle compounds was very low.

^{14}C1-Glucose versus ^{14}C6-Glcuose metabolism. Metabolism via the Embden-Meyerhof pathway yields CO_2 from glucose from both the C1 position and the C6 position in equal amounts. Glucose metabolism via the oxidative pentose phosphate pathway yields CO_2 initially only from the C1 position. In order to find the contribution of these pathways to glucose metabolism in R. sphaeroides, I performed the following experiment.

Wild-type and Glc$^+$ strains were grown with glucose as substrate until H_2 production was observed in the Glc$^+$ cultures. ^{14}C1-glucose was added to half of the cultures of each type. ^{14}C6-glucose was added to each of the remaining cultures. The $^{14}CO_2$ released into the culture media and gas phases was collected and counted. Data appear in Table 4-4. The ratio of C1 position label versus C6 position label was about one throughout the experiment for wild-type cells grown on glucose. Glc+ strain cells cultured on glucose initially showed ratios of about 1.5, which decreased over time to about one.

^{14}C-$NaHCO_3$ Fixation in Photoheterotrophic Cultures. In a closed culture system, cultures photoproducing H_2 rapidly create a reducing environment rich in CO_2. It seemed worthwhile to determine the extent and pathways of CO_2 fixation in R. sphaeroides growing photoheterotrophically and whether this diminished H_2 production.

Wild-type and Glc$^+$ strain cultures were grown photoheterotropically on malate and glucose respectively until H_2 production was observed. Ezymatic inhibitors were added to some cultures. Ammonium ion was used to supress H_2 production. Carbon monoxide was used to block the generation of reducing power from H_2 via hydrogenase. Fluoroacetate was used to block cis-aconitase and prevent the operation of the TCA cycle. ^{14}C-$NaHCO_3$ or ^{32}P-phosphate and cold bicarbonate were used in the tracer studies. Data appeared in Figs. 4-12 to 4-26.

^{14}C-Bicarbonate Fixation in the Wild-type Strain. Wild-type cells photoproducing H_2 from malate fixed ^{14}C-bicarbonate about 0.5% relative to the amount of CO_2 produced from malate metabolism. Carbon monoxide did not appear to have a significant effect. The addition of fluoroacetate increased total ^{14}C-bicarbonate fixation two to threefold. The addition of ammonium ion stimulated fixation tenfold.

The primary labeled pool was the insoluble material, primarily polyglucose, which accounted for 70% of the acid-stable counts. Malate, fumarate and gluconate were the most heavily labeled intermediates. ^{14}C-label also appeared in the C5 acid, ATP, AMP and glycolate. In cultures labeled with ^{32}P-phosphate, about 80% of the label fixed was found in the insoluble material, 9% was in glucose-6-phosphate and 5% was in 6-phosphogluconate. Fructose-6-phosphate and the adenylates were also labeled. The energy charge was signficantly lower during H_2 production by these cells and increased as substrate was depleted.

The addition of fluoroacetate yielded strong labeling of citrate as well as a fourfold increase in labeled pools of malate and fumarate. α-ketoglutarate and phosphoenolpyruvate also became labeled. ^{32}P-labeled

pools had a general threefold decrease in labeling, except for the polymers, which increased slightly. Although the level of adenylate labeling decreased, the energy charge of the cells remained unchanged.

In cultures grown at H_2-inhibiting levels of ammonium ion, the pattern and amount of labeling is significantly different. General fixation increases tenfold over the system producing H_2 (Table 4-5). However, glutamate increases 100-fold and both aspartate and glycolate show 50-fold increases. The amount of label in insoluble material increases sixfold, but the relative percentage of label found in this pool declined.

Wild-type cells grown on glucose, CO_2 and ^{32}P-phosphate showed a doubling of label in insoluble materials, 6-phosphogluconate, fructose-6-phosphate and the adenylates relative to cultures with CO_2 removed. The addition of fluoroacetate gave a reduction in labeling in 6-phosphogluconate and an increase in fructose-6-phosphate labeling.

^{14}C-Bicarbonate Fixation in the Glc$^+$ Strain. The Glc$^+$ strain grown on malate and ^{14}C-bicarbonate showed patterns of ^{14}C-labeling similar to the wild-type, except that label in gluconate and C5 acid were several-fold lower. ^{32}P-labeling from ^{32}P-phosphate was primarily into insoluble material and was fourfold less than with the wild-type strain. Labeling in fructose-6-phosphate was 50% that found with the wild-type. The other intermediates, including 6-phosphogluconate and glucose-6-phosphate, had little or no ^{32}P-labeling.

The addition of fluoroacetate to cultures grown on malate and ^{14}C-bicarbonate yielded an increase in ^{14}C-gluconate and ^{14}C5 acid. ^{32}P-labeling doubled in the insoluble material and disappeared from fructose-6-phosphate. Other pools maintained their labeling levels.

When the Glc$^+$ strain was grown on glucose and ^{14}C-bicarbonate, fixation of ^{14}C was only about 1% of the CO_2 produced from glucose metabolism. Acid-stable fixation was generally increasing with time except for cultures containing ammonium ion, which were initially large and decreased with time. Ammonium ion stimulated total fixation tenfold. Carbon monoxide did not seem to have a significant effect on total fixation or relative pool size.

Almost 90% of the ^{14}C-label fixed appeared in the insoluble material, which was primarily polyglucose. Gluconate and C5 acid, malate, glycolate, ATP, fumarate, glycerate and AMP showed labeling in decreasing order. About 80% of the ^{32}P-label appeared in insoluble material. Ten percent was found in fructose-6-phosphate, 5% in glucose-6-phosphate and the remainder in 6-phosphogluconate and the adenylates.

Addition of fluoroacetate to this system resulted in heavy labeling of citrate. This became the dominant pool with 60-70% of the ^{14}C-bicarbonate fixed. Insoluble material and phosphoenolpyruvate each had about 6% of the total ^{14}C-label. Lesser amounts of ^{14}C-label were found in glycolate, α-ketoglutarate, malate, gluconate, C5 acid and glycerate. Fluoroacetate gave a threefold increase in ^{32}P-labeling of insoluble material and a fivefold increase in 6-phosphogluconate. ^{32}P-fructose-6-phosphate pools declined.

When H_2 production was inhibited with ammonium in Glc$^+$ strain cultures growing on glucose, ^{14}C-bicarbonate fixation increased tenfold (Table 4-5). 85% of the fixed material was found in the insolubles. ^{14}C-label in glycolate increased 20-fold and was 5% of the total. Gluconate and C5 acid doubled the amount of their labeling with the

addition of ammonium ion. Glycerate, fumarate and malate each contained about 1% of the fixed label.

Analysis of Storage Material. Insoluble material labeled with ^{14}C or ^{32}P from the above experiments was hydrolyzed in boiling aqueous acid, neutralized and characterized in the same manner as was previously described. It was found that regardless of the type-strain of *R. sphaeroides* or the ^{14}C-carbon source employed, ^{14}C-label was found in glucose, alanine and material that did not hydrolyze. Except for cultures that contained ammonium ion, glucose was the primary material in the hydrolyzate. Generally, glucose accounted for more than half of the label. β-hydroxybutyrate was not observed. When ammonium ion was present, hydrolyzates contained alanine, glutamine and glutamate in significant amounts and the amount of label in glucose was reduced fivefold. Hydrolysis of ^{32}P-labeled insoluble material yielded exclusively ^{32}P-phosphate.

DISCUSSION

The intention of this series of experiments was to let the patterns of radioactive labeling from the various substrates suggest the metabolic pathways involved. For the most part, the experiments yielded patterns from long-term steady state labeling which allowed calculation of the stoichiometry and efficiency of metabolism under the different culture conditions. The determination of intermediates and end-products suggested the *in vivo* regulation pattern of substrate distribution and shed light on the role of H_2 production for metabolic balance in *R. sphaeroides*.

For R. sphaeroides in general, metabolism of a substrate utilizable for growth resulted in a stoichiometric production of CO_2. No other compound appeared to accumulate stoichiometrically. In the specific instance of wild-type R. sphaeroides cultured on glucose, gluconate was formed in near stoichiometric amounts, although growth under these conditions was low (Table 4-1). In cases where the organism was capable of H_2 production, H_2 was formed in nearly stoichiometric amounts once the culture began producing it.

The material that was insoluble in the chromatographic solvents and which remained at the origin was primarily polyglucose, since the hydrolyzate of the ^{14}C-labeled origins was mostly ^{14}C-glucose. No β-hydroxybutyrate was observed. This is consistent with the work of Stainer, et al. (73), which claimed that R. rubrum stored carbon as β-hydroxybutyrate when grown on acetate, but stored carbon as polysaccharide when grown on succinate or CO_2. It appears that the particular storage material may be a function of the primary carbon source.

Although ammonia levels approach zero before H_2 production is initiated, amino acid levels need not be low for nitrogenase to be active. When cells were cultured on minimal medium and 5 mM glutamate, H_2 production began when glutamate levels were 2-3 mM. However, little ^{14}C-label becomes incorporated into protein. Some ^{14}C-alanine was recovered from hydrolyzed origin material, but little free ^{14}C-alanine was observed. Alanine probably arises from the reaction glutamate + pyruvate = α-ketoglutarate + alanine.

Even with a significant amount of glutamate present during H_2 production, ^{14}C-labeling of amino acids was nil. Transamination to

pyruvate may yield alanine. Transamination to oxaloacetate to produce aspartate was not observed, nor was the production of glutamine. This also eliminates the participation of stoichiometric amounts of glutamate in metabolism of other substrates. While some pyruvate may be used up in alanine synthesis, alanine in not a major end-product.

Metabolic Pathways of the Wild-type Strain. Long-term ^{14}C- and ^{32}P-labeling studies with malate or glucose as carbon sources suggested that the tricarboxylic acid (TCA) cycle functions to produce reducing power in *R. sphaeroides*, and that it is not the only pathway available to the organism for this. When malate was provided as a carbon source and the TCA cycle blocked at cis-aconitase with fluoroacetate, malate was still metabolized and H_2 production continued, although at lower rates and yields. Citrate accumulated with time, suggesting that the TCA cycle was blocked, although the completeness of this block was not determined. Some form of gluconeogenesis must occur, since glucose and fructose appeared early and the insoluble material was primarily polyglucose. Labeling in glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, phosphoglycerate and phosphoenolpyruvate support this as well.

Glucose was poorly metabolized by the wild-type. Little substrate was utilized, growth was limited and no H_2 was produced. When ^{14}C-glucose was fed to this strain, the predominant labeled pools were polyglucose and gluconate, suggesting that gluconate was an end-product of glucose metabolism in the wild-type. Gluconate, and to a lesser extent, the C5 acid were significantly labeled in wild-type cultures growing on ^{14}C-malate.

Gluconate could arise from glucose via a direct oxidation, or more traditionally, via oxidation of glucose-6-phosphate to 6-phosphogluconate, then dephosphorylation to gluconate. This is coupled in metabolism in the oxidative pentose phosphate pathway with the generation of NADPH. Work by Szmona and Doudoroff suggest that both pathways to gluconate are possible (76).

It appears as if there is a partitioning of substrate by R. sphaeroides. With malate as the substrate, 90% of the material is metabolized to yield H_2 and CO_2. The remaining material is routed into storage material, primarily polyglucose, or to a lesser extent into gluconate. When fluoroacetate inhibits TCA cycle metabolism, the proportion of the material flowing into gluconate and polymer increases. When glucose is the substrate, growth is limited. Movement of substrate into gluconate or into polymer is enhanced relative to metabolism to CO_2. Fluoroacetate further diminishes metabolism, but conversion of substrate gluconate or polymer is relatively unchanged.

Metabolic Pathways of the Glc^+ Strain. There were significant differences in labeling patterns between the wild-type strain and the Glc^+ strain. Glc^+ strain cells grown on ^{14}C-glucose has a 15-fold decrease in relative ^{14}C-gluconate labeling. A higher proportion of the substrate was stored as polyglucose. This material was accessible to the cell, since it disappeared when the substrate was depleted. Glycolate appeared at much higher levels than with the wild-type and appeared to be associated with glucose metabolism.

Szymona and Doudoroff described hexose metabolism in wild-type R. sphaeroides as occurring only poorly, with the accumulation of aldonic acids and 2-keto-3-deoxygluconic acid (76). The Embden-Meyerhof pathway was considered constitutive, but limited due to low aldolase activity. They found mutant strains capable of increased glucose utilization and described the Entner-Doudoroff pathway for hexose metabolism. The major steps in this system were the conversion of 6-phosphogluconate to 2-keto-3-deoxygluconate and the cleavage of this compound to glyceraldehyde-3-phosphate and pyruvate. Conrad and Schlegel, using these mutant strains, demonstrated a high level of activity in the enzymes of the Entner-Doudoroff pathway when the cells were growing anaerobically on glucose or fructose (17). In differential radiorespirometry experiments using ^{14}C- and $^{14}C_6$-labeled hexoses, initial rates of $^{14}CO_2$ evolution were 20-40 times higher from the $^{14}C1$-glucose as from the $^{14}C6$-glucose. They concluded that all sugar catabolism was via the Entner-Doudoroff pathway.

My own data do not support this. $^{14}C1$- versus $^{14}C6$-glcuose differential radiorespirometry experiments with the wild-type and Glc$^+$ strains showed $^{14}C1$: $^{14}C6$ ratios of unity for wild-type cultures and approximately 1.5: 1 for Glc$^+$ strain cultures. This suggests that glucose catabolism via the Embden-Meyerhof pathway is significant in this organism. There is no particular evidence to support the operation of a complete Entner-Doudoroff pathway. Glyceraldehyde-3-phosphate and pyruvate were not appreciably labeled. Although glutamate was present in millimolar quantities, a reaction of this and pyruvate to yield alanine was not significant. 2-keto-3-deoxygluconate or 2-keto-3-deoxy-6-phosphogluconate were not observed. The standard sugar mono- and diphosphates of the

Embden-Meyerhof pathway appeared labeled in most cases. Supporting this also are the observations that levels of NAD^+ + NADH were about three-times those of $NADP^+$ + NADPH, suggesting a larger contribution of glycolysis versus the oxidative pentose phosphate pathway or the Entner-Doudoroff pathway. Thus, it appears that the $\underline{Glc}^+$ strain is not similar to the glucose-utilizing strains used by others. Since the glycolytic enzyme aldolase has been reported to be the limiting enzyme for hexose metabolism via the Embden-Meyerhof pathway in _R. sphaeroides_, the $\underline{Glc}^+$ may be enhanced for this enzyme.

Partitioning of glucose between storage and catabolism appeared to be different in the $\underline{Glc}^+$ from the wild-type. While the relative amount of substrate material accumulating in polyglucose was the same for both strains, substrate flow into gluconate, C5 acid and 6-phosphogluconate was strongly reduced with the $\underline{Glc}^+$ strain. More material was completely metabolized to CO_2 and H_2. If metabolism through gluconate and the Entner-Doudoroff pathway is minimal, then accumulation of substrate into gluconate would be as an end-product. This would appear to be wasteful in terms of generation of reducing power. On the other hand, substrate material flowing into polyglucose appeared to be recoverable. As substrate was depleted, material in the origins began to disappear.

The appearance of glycolate is not easily explainable. Glycolate is a product of photorespiration, where ribulose 1,5 biphosphate combines with O_2 rather than with CO_2. The system used here with _R. sphaeroides_ is anaerobic and would be expected to have a minimal level of photorespiration. The glyoxylate cycle might be responsible for the observed glycolate. Eley, _et al_. reported a complete glyoxylate cycle for _R. palustris_

(23). However, this pathway was active only on culturing with acetate as substrate and may be generally associated with fatty acid catabolism. Succinate, a primary product of the glyoxylate cycle, was not significantly labeled from ^{14}C-substrates in any case with R. sphaeroides. Anderson and Fuller report that CO_2 fixation in the presence of malate yields glycolate as an initial product of fixation in R. rubrum (2,3a). Fixation of CO_2 by R. sphaeroides under the conditions allowing H_2 production was low, yet may account for the observed amounts of glycolate. Why this would be significantly higher in the Glc^+ strain is unclear.

CO_2 Fixation During H_2 production in Photoheterotrophic Cultures. It was of interest to see the amount of CO_2 released in catabolism that was refixed by R. sphaeroides. During H_2 production, stoichiometric amounts of CO_2 are released and the atmosphere by virtue of H_2 becomes a reducing one. It has been reported that under these conditions, R. sphaeroides can grow autotrophically with the fixation of CO_2 with reductants generated from H_2 (3). Large amounts of CO_2 fixation would "eat up the profits" of H_2 biosynthesis, significant from an economic viewpoint, and alter the levels of metabolic intermediates, which would affect the evaluation of the contributions of the various degradative pathways. The amount of ^{14}C-bicarbonate fixed when wild-type or Glc^+ strain cells were producing H_2 was 1% or less of the CO_2 released from substrate metabolism. This supports the idea that the labeled compounds seen on chromatograms resulted directly from labeled substrates.

The majority of the fixation product was found in insoluble material and appeared to be polyglucose both with the wild-type and with the Glc^+ strain. Malate and fumarate had significant label in all cases. Wild-

type cultures accumulated gluconate, 6-phosphogluconate and C5 acid as well. The hexose monophosphates had labeling in all cases. Glycolate levels in cultures producing H_2 were low. Addition of ammonium ion increased fixation tenfold and label into glycolate increased 20-50-fold.

Anderson and Fuller (3a,b) demonstrated the Calvin cycle in R. rubrum grown autotropically. In photoheterotrophic cultures, however, the initial CO_2 fixation product was glycolate (2). These cultures were grown with wild-type cells, malate and ammonium ion. The data of my experiments support the role of glycolate as a primary product of CO_2 fixation in R. sphaeroides cultures under the same conditions, and suggest that other fixation routes are active as well.

Under conditions allowing H_2 production, with minimal ammonium ion present, the principal labeled compounds are malate and fumarate, even when malate is the substrate and is present at high concentration. A possible explanation is a fixation of CO_2 by phosphoenolpyruvate (PEP) carboxylase to yield oxaloacetate, which could then be reduced to malate and fumarate. With a large initial pool of malate, ^{14}C-malate from $^{14}CO_2$ fixation would be trapped in the unlabeled material and persist. The ready equilibrium with fumarate would tend to allow accumulation of label in that pool as well. The energetics for the PEP carboxylase reaction, proposed by Paul, et al. (65), are highly favorable for the generation of oxaloacetate in vivo. The reaction of oxaloacetate to malate, even with the low observed $NADH/NAD^+$ ratio and the probable high (malate)/(oxaloacetate) ratio, is still exothermic for synthesis of malate and would be expected to proceed in that direction.

L-malate + NAD^+ = oxaloacetate + NADH $G° = 7.1$

Assume $(NADH)/(NAD^+) = 1$ and (malate)/(oxaloacetate) = 10,000

$7.1 - RT \ln 10^4 = 1.7$

Buchanan et al. (10,11) suggest that there is a ferredoxin-dependent reversal of the TCA cycle responsible for CO_2 fixation in R. rubrum and other photosynthetic bacteria. They identify glutamate as the predominant initially labeled product from ^{14}C-bicarbonate in cultures grown on malate or acetate and ammonium ion. Presumably this is from fixation of CO_2 by succinate to yield α-ketoglutarate, which may pickup an amino group to yield glutamate. Aspartate, which could arise from amination of oxaloacetate, was also significantly labeled under these conditions. My own work with R. sphaeroides under these conditions was consistent with their observations. In wild-type cells grown on malate and ammonium ion, label from ^{14}C-bicarbonate appeared heavily in glutamate (28% of the total label fixed) and in aspartate (13% of the total label fixed). However, these compounds had less than 1% each of the fixed label with the Glc^+strain under the same conditions. In cultures producing H_2, there was no evidence to support a reductive TCA cycle. In the presence of fluoroacetate, citrate was labeled tenfold more than was α-ketoglutarate from ^{14}C-bicarbonate. This suggests that the TCA cycle was not in fact cycling, although bicarbonate was being fixed. It does not support a significant fixation of CO_2 by succinate, since α-ketoglutarate levels were relatively low.

There appeared to be little or no contribution to CO_2 fixation from the Calvin cycle or into glycolate under conditions allowing H_2 formation. In any case, fixation was minimal relative to the amount of CO_2 produced

by substrate metabolism. Carbon flow of fixation products appeared to follow the patterns of carbon flow associated with the particular strains and culture conditions. Most labeled material accumulated as polyglucose. Since this storage product seemed to be accessible to R. sphaeroides, particularly to the Glc^+ strain after substrate depletion, the practical loss of substrate for H_2 production should be minimal.

TABLE 4-1. Stoichiometry of H_2 Production

Wild-type and Glc$^+$ R. sphaeroides were grown in minimal medium with 20 mM (100 umole) carbon substrate anaerobically in the light. After the initiation of H_2 production, the appropriate ^{14}C-labeled substrates were added. When fluoroacetate was added, it was added one hour before the tracer. H_2 was quantified manometrically. $^{14}CO_2$ was counted by scintillation. Other compounds were separated by paper chromatography and counted by Geiger counter.

Strain	Substrate	Fluoro-acetate	H_2[b]	CO_2[2]	Gluconate[b]	Citrate[b]	Insoluble[b]
W.T.	malate	-	360	380	0	0	4.3
W.T.	"	+	42	120	3.1	0.03	41
W.T.	glucose	-	0	15	9.0	0	5.0
W.T.	"	+	0	12	7.0	0.02	7.0
Glc$^+$	malate	-	330	380	0	0.03	21.1
Glc$^+$	"	+	60	160	0	0.58	4.1
Glc$^+$	glucose	-	240	150	1.6	0.005	26
Glc$^+$	"	+	120	150	3.9	0.11	24

(a) 5 mM fluoroacetate was added to some cultures as an inhibitor of the tricarboxylic acid cycle.

(b) Quantities are μmoles.

TABLE 4-2. Effect of Fluoroacetate on Rate and Conversion of Substrate to H_2

The appropriate strains of R. sphaeroides were grown anaerobically in minimal medium with 20 mM of the indicated carbon substrate in the light. H_2 was quantified manometrically. Fluoroacetate when added was added before H_2 production began.

Strain	Substrate	Fluoroacetate concentration (mM)				
		0	1	2	5	10
W.T.	malate	3.6 (62)[a]	3.0	1.5 (37)	1.2 (19)	1.2 (16)
W.T.	glucose	0	--	--	0	--
W.T.	fructose	3.3 (12)	2.2	2.0 (9)	--	2.0 (8)
W.T.	lactate	3.2 (38)	--	1.8 (14)	2.0 (15)	1.8 (14)
Glc$^+$	malate	3.7 (57)	3.6 (43)	--	2.4 (24)	2.5 (23)
Glc$^+$	glucose	4.0 (80)	--	2.1 (47)	2.1 (40)	1.9 (35)
Glc$^+$	fructose	2.7 (22)	--	1.8 (16)	1.8	--
Frc$^+$	fructose	3.7 (55)	--	1.9	1.6 (41)	1.5

(a) Values are ml H_2 evolved per hr per mg protein, and (percent of substrate converted to H_2).

TABLE 4-3. Fractionation of Unknown Acids by pK on Dowex 1 Columns

The ^{14}C-labeled eluents of paper chromatogram spots co-chromatographing in water-saturated phenol (pH 4.3) and butanol: propionic acid with aspartate and glutamate were layered onto acetic acid charge Dowex 1 columns and eluted sequentially with water (pH 5.9), 2N acetic acid (pH 2.4), formic acid (pH 1.8), 2N HCl (pH 0). Eluent samples were counted by scintillation.

Material	Associated Spot	Dowex 1 fractions				Estimated
		H_2O	Acetic acid	Formic acid	HCl	pKa
unknown A	aspartate	345	348	3142	17133	1.1
unknown B	glutamate	55	27	598	60	2.1
gluconate	aspartate	564	241	933	7459	1.0

TABLE 4-4. $^{14}CO_2$ Released from Differentially Labeled *R. sphaeroides*

Wild-type and *Glc*$^+$ strain cells were grown anaerobically on minimal medium with 30 mM glucose in the light. After the initiation of H_2 production in the *Glc*$^+$ cultures, ^{14}C-glucose labeled in the C1 or C6 position was added to all tubes. Medium and atmospheric $^{14}CO_2$ were collected in Warburg flasks and counted by scintillation.

Strain	Time point [a]	$^{14}CO_2$ from ^{14}C1-glucose [b]	$^{14}CO_2$ from ^{14}C6-glucose [b]	C1/C6 ratio
W.T	t= 1	98,000	126,000	0.8
	t= 2	228,000	206,000	1.1
	t= 4	321,000	303,000	1.1
Glc$^+$	t= 1	99,000	72,000	1.4
	t= 2	296,000	181,000	1.6
	t= 4	507,000	453,000	1.1

(a) Time after ^{14}C-label addition in hrs.

(b) Counts per min.

TABLE 4-5. Effect of Ammonium on ^{14}C-Bicarbonate Fixation

Wild-type cells with 40 mM malate and Glc$^+$ cells with 40 mM glucose were grown in minimal medium anaerobically in the light. After the initiation of H_2 production, ^{14}C-bicarbonate was added. Some cultures received 5 mM NH_4Cl to inhibit H_2 production. Samples were assayed by paper chromatography and autoradiography.

	Wild-type strain		Glc$^+$ strain	
Material	Label increase	% total fixed	label increase	% total fixed
Total fixation	10X[a]	-	10X[a]	-
Insolubles	6X	40%	10X	85%
Glycolate	50X	5%	20X	5%
Glycerate	10X	1%	6X	1%
Adenosine-monophosphate	10X	<1%	10X	<1%
Adenosine-triphosphate	10X	2%	10X	<1%
Glutamate	100X	28%	2X	1%
Aspartate	50X	13%	2X	1%
Phosphoenolpyruvate	20X	2%	--	--
α-ketoglutarate	--	--	--	--
Citrate	--	--	10X	<1%
Fumarate	10X	3%	10X	1%
Malate	10X	7%	10X	1%

(a) Increase in labeling in pools of cultures with ammonium ion versus control cultures without ammonium ion.

FIGURE LEGENDS

4-1 15 ml serum-capped side-arm tube. Side arm contained solid NaOH.

4-2 ^{14}C-labeled insoluble material from ^{14}C-substrate metabolism. Wild-type and mutant strain cultures were grown on 20 mM of the indicated carbon sources anaerobically in the light. After the general initiation of H_2 production, the appropriate ^{14}C-labeled carbon sources were added. When 5 mM fluoroacetate was added, it was added one hour before the ^{14}C-labeled material. Samples were assayed by paper chromatography, autoradiography and Geiger-counting (see Methods, Chapter Two for details). Values are µgrams of compound per mgram protein based on the specific activity of the starting ^{14}C-labeled carbon source. Symbols: ○-○, ^{14}C-malate as substrate; ●-●, ^{14}C-malate as substrate, with fluoroacetate; △-△, ^{14}C-glucose as substrate; ▲-▲, ^{14}C-glucose as substrate, with fluoroacetate; □-□, ^{14}C-lactate as substrate; ■-■, ^{14}C-lactate as substrate, with fluoroacetate; ◇-◇, ^{14}C-fructose as substrate; ◆-◆, ^{14}C-fructose as substrate, with fluoroacetate.

4-3 ^{14}C-labeled malate from ^{14}C-substrate metabolism. Methods and symbols as in Fig. 4-2.

4-4 ^{14}C-labeled fumarate from ^{14}C-substrate metabolism. Methods and symbols as in Fig. 4-2.

4-5 ^{14}C-labeled citrate from ^{14}C-substrate metabolism. Methods and symbols as in Fig. 4-2.

4-6 ^{14}C-labeled phosphoenolpyruvate from ^{14}C-substrate metabolism. Methods and symbols as in Fig. 4.2.

4-7 ^{14}C-labeled 6-phosphogluconate from ^{14}C-substrate metabolism. Methods and symbols as in Fig. 4-2.

4-8 ^{14}C-labeled gluconate from ^{14}C-substrate metabolism. Methods and symbols as in Fig. 4-2.

4-9 ^{14}C-labeled "C5 acid" from ^{14}C-substrate metabolism. Methdos and symbols as in Fig. 4-2.

4-10 ^{32}P-labeled insoluble material and 6-phosphogluconate from ^{32}P-phosphate labeling. Wild-type and Glc$^+$ strain cells were grown on 20 mM of the indicated carbon sources and minimal medium anaerobically in the light. After the general onset of H_2 production, ^{32}P-phosphate was added to all cultures. When 5 mM fluoroacetate was added, it was added one hour before the ^{32}P-phosphate. Samples were assayed by paper chromatography and Geiger-counting. Values are μgrams of compound per mgram protein. Symbols: ○-○, ^{14}-malate as substrate; ●-●, ^{14}C-malate as substrate, with fluoroacetate; △-△, ^{14}C-glucose as substrate; ▲-▲, ^{14}C-glucose as substrate, with fluoroacetate, (A) ^{32}P-labeled insoluble material. (B) ^{32}P-labeled 6-phosphogluconate.

4-11 ^{32}P-labeled glucose-6-phosphate and fructose-6-phosphate. Methods and symbols as in Fig. 4-10. (A) ^{32}P-labeled glucose-6-phosphate. (B) ^{32}P-labeled fructose-6-phosphate.

4-12 Total fixation from ^{14}C-bicarbonate in H producing R. sphaeroides. Wild-type cells with 20 mM malate and Glc$^+$ strain cells with 20 mM glucose were grown in minimal medium anaerobically in the light. After the initiation of H_2 production, ^{14}C-labeled $NaHCO_3$ (5 mM) was added to all cultures. When 5 mM fluoroacetate or 10% (v/v) carbon monoxide

were added, they were added one hour before the bicarbonate. Samples were assayed by paper chromatography and Geiger-counting. Values were gram-atoms of carbon fixed per mgram protein. Symbols: ○-○, control; ●-●, 10% carbon monoxide; △-△, 5 mM fluoroacetate; ▲-▲, 5 mM fluoroacetate and 10% carbon monoxide.

4-13 ^{14}C-baleled insoluble material from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-12.

4-14 ^{14}C-labeled malate from ^{14}C-bicarbonate fixation. Values are ugrams of compound per mgram protein. Methods and symbols as in Fig. 4-12.

4-15 ^{14}C-labeled fumarate from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-14.

4-16 ^{14}C-labeled α-ketoglutarate from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-14.

4-17 ^{14}C-labeled citrate from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-14.

4-18 ^{14}C-labeled phosphoenolpyruvate from ^{14}C bicarbonate fixation. Methods and symbols as in Fig. 4-14.

4-19 ^{14}C-labeled glycerate from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-14.

4-20 ^{14}C-labeled glycolate from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-14.

4-21 ^{14}C-labeled gluconate from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-14.

4-22 ^{14}C-labeled "C5 acid" from ^{14}C-bicarbonate fixation. Methods and symbols as in Fig. 4-14.

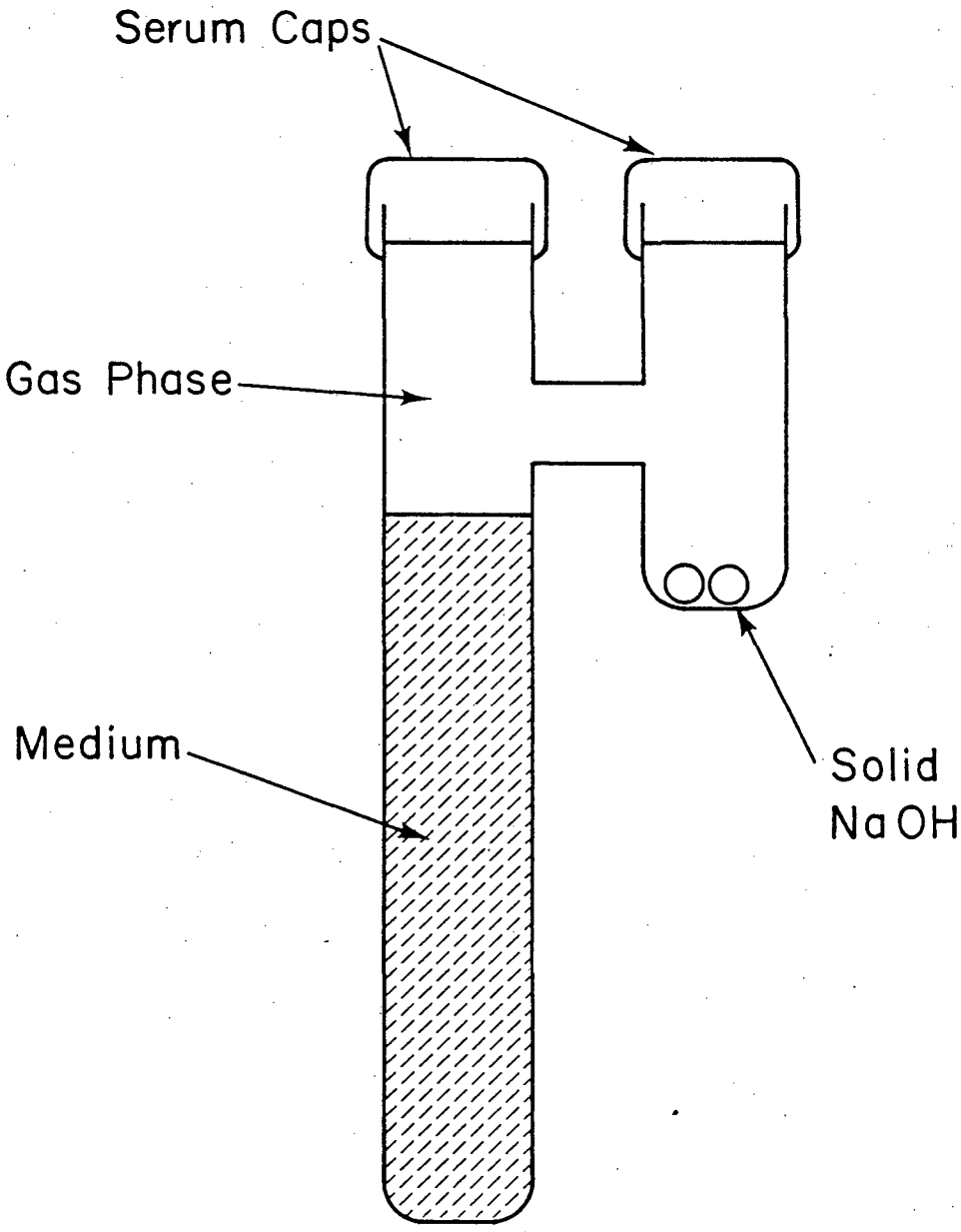
4-23 ^{32}P-labeled insoluble material and 6-phosphogluconate from ^{32}P-phosphate during bicarbonate fixation. Wild-type and Glc$^+$ strain cells on 20 mM glucose or malate were grown in mininmal medium anaerobically in the light. After the initiation of H_2 production, 10 mM $NaHCO_3$. then ^{32}P-phosphate (1 mM) were added to all cultures. When 5 mM fluoroacetate was used, it was added one hour before the bicarbonate and phosphate. Samples were assayed by paper chromatography and Geiger-counting. Values are µgrams-atoms of bicarbonate fixed per mgram protein for the insoluble material and µgrams compound per mgram protein for 6-phosphogluconate. Symbols: ○-○, malate substrate; ●-●, malate substrate, with fluoroacetate; △-△, glucose substrate; ▲-▲, glucose substrate, with fluoroacetate. (A) ^{32}P-labeled insoluble material. (B) ^{32}P-labeled 6-phosphogluconate.

4-24 ^{32}P-labeled glucose-6-phosphate and fructose-6-phosphate from ^{32}P- phosphate during bicarbonate fixation. Methods and symbols as in Fig. 4-23. (A) ^{32}P-labeled glucose-6-phosphate. (B) ^{32}P-labeled fructose- 6-phosphate.

4-25 ^{32}P-labeled adenosine triphosphate and adenosine diphosphate from ^{32}P-phosphate during bicarbonate fixation. Methods and symbols as in Fig. 4-23. (A) ^{32}P-labeled adenosine triphosphate. (B) ^{32}P-labeled adenosine diphosphate.

4-26 ^{32}P-labeled adenosine monophosphate from ^{32}P-phosphate during bicarbonate fixation. Methods and symbols as in Fig. 4-23.

Fig. 4-1 102



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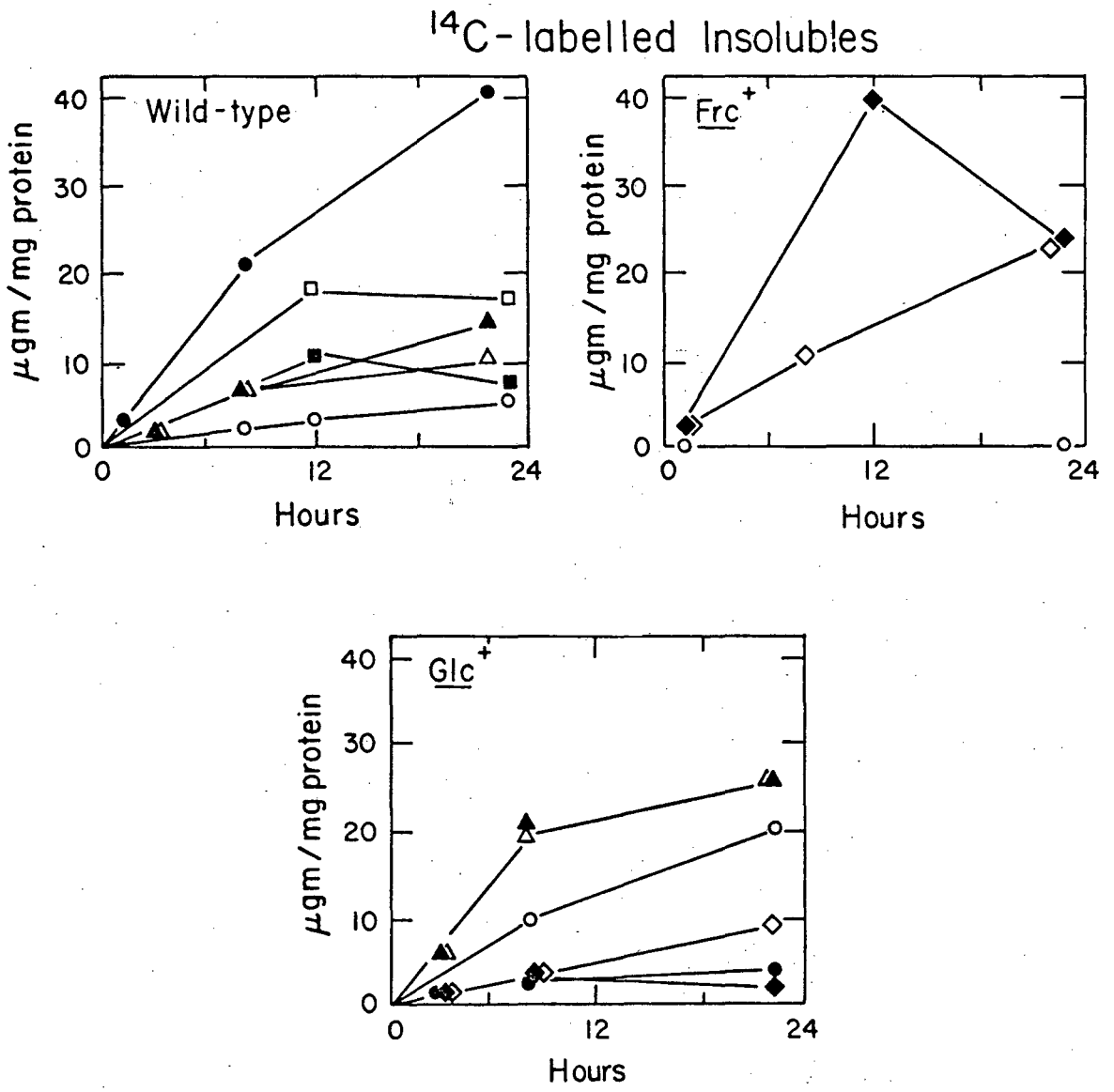

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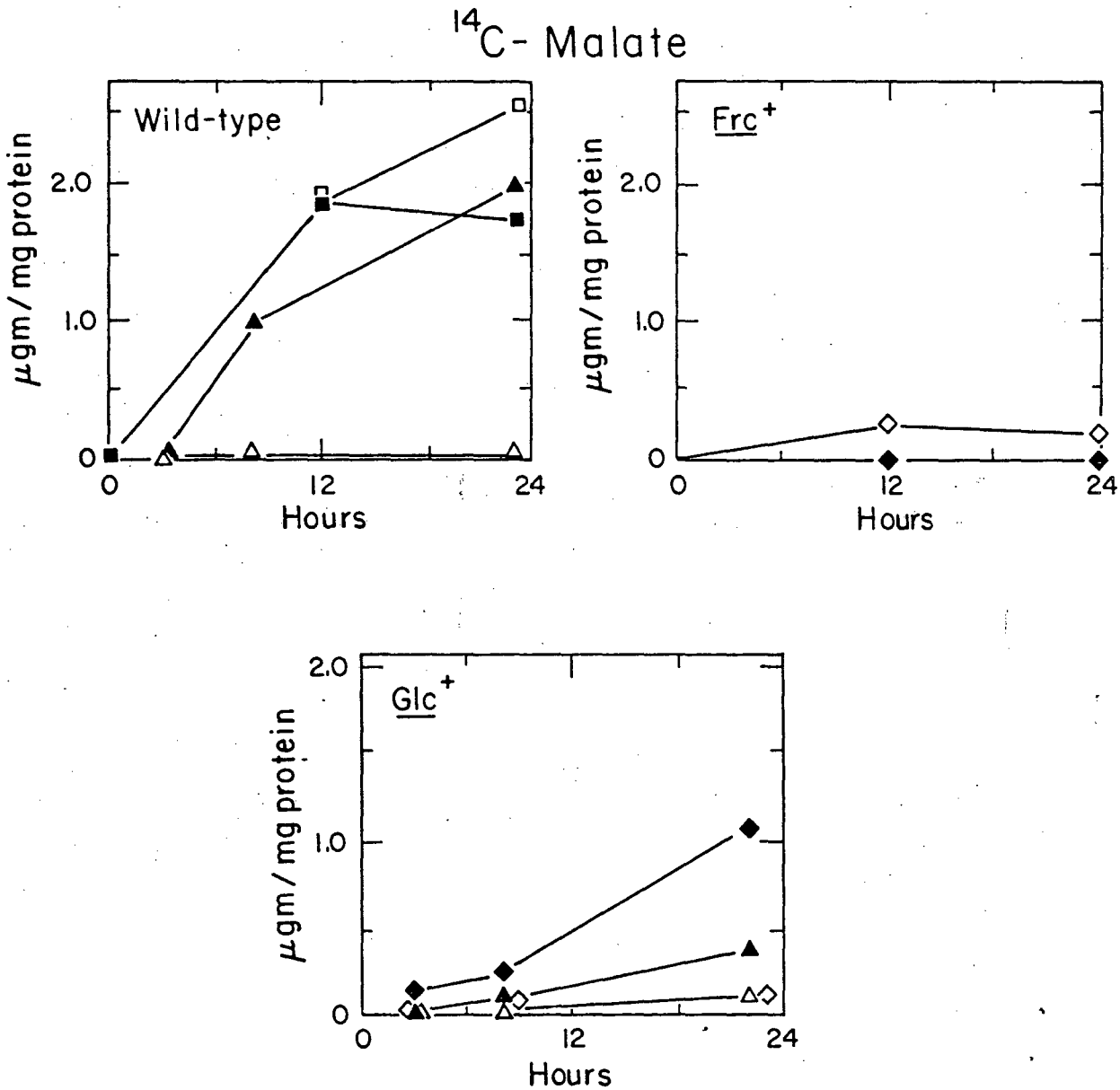

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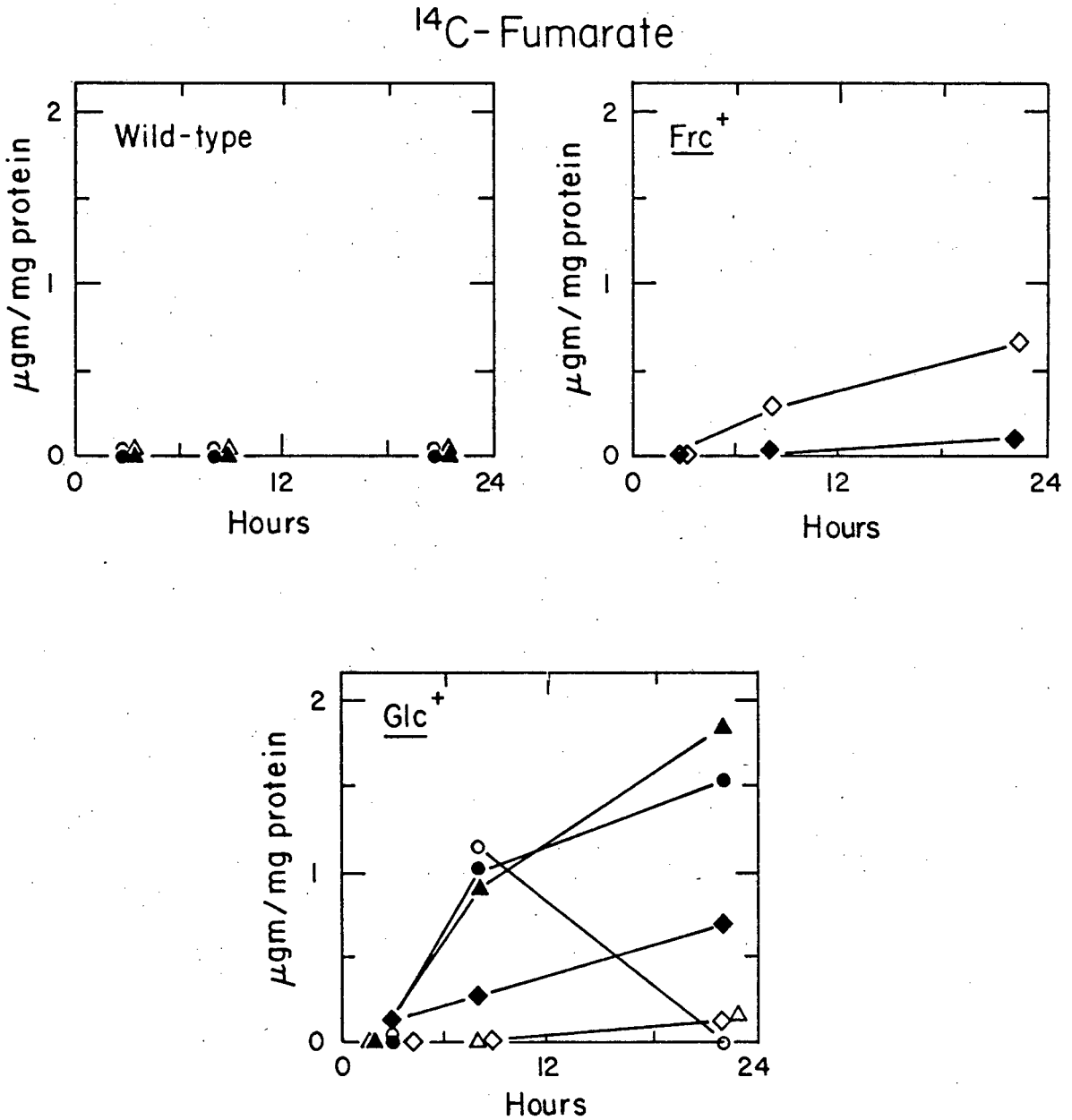

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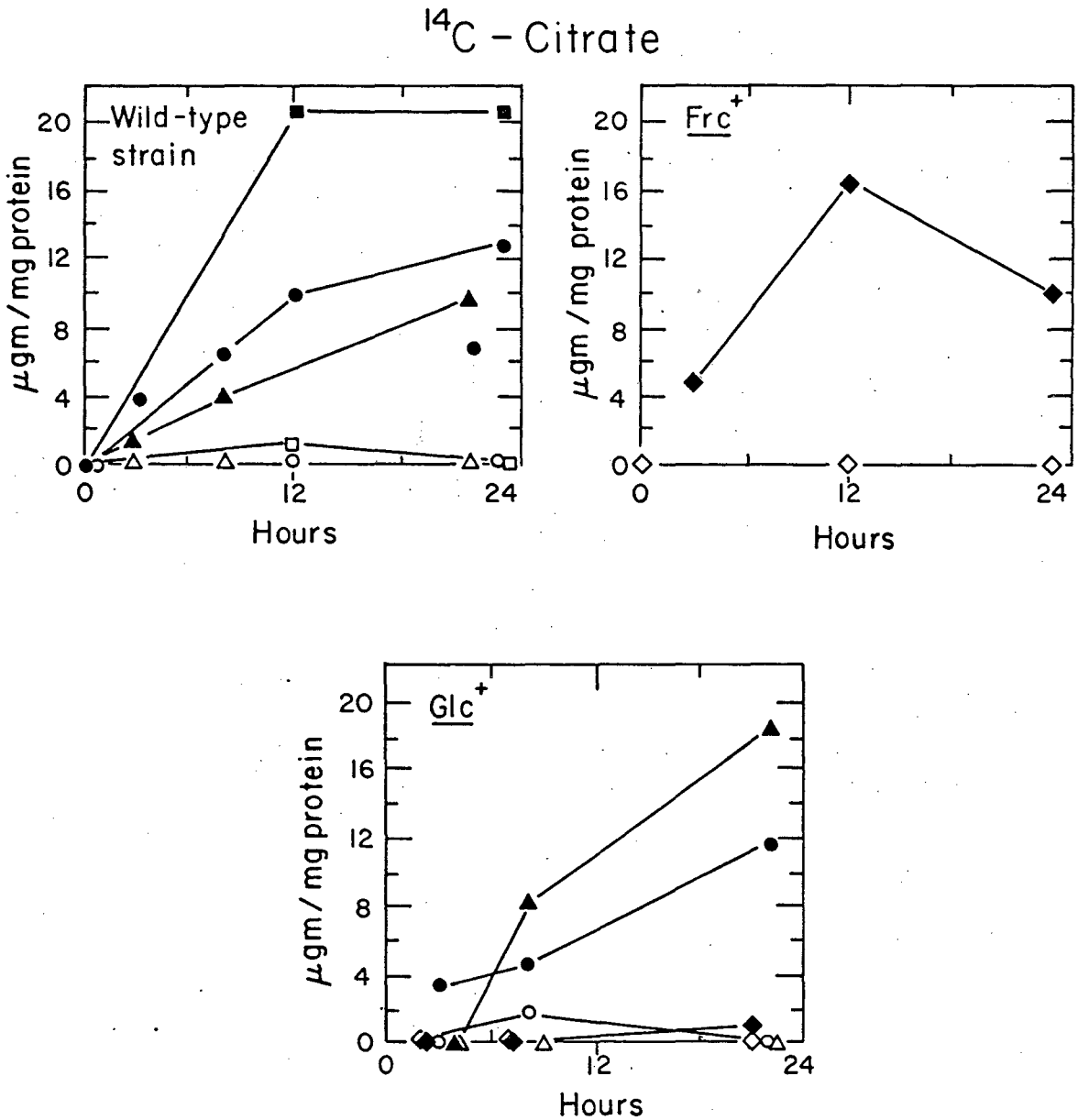

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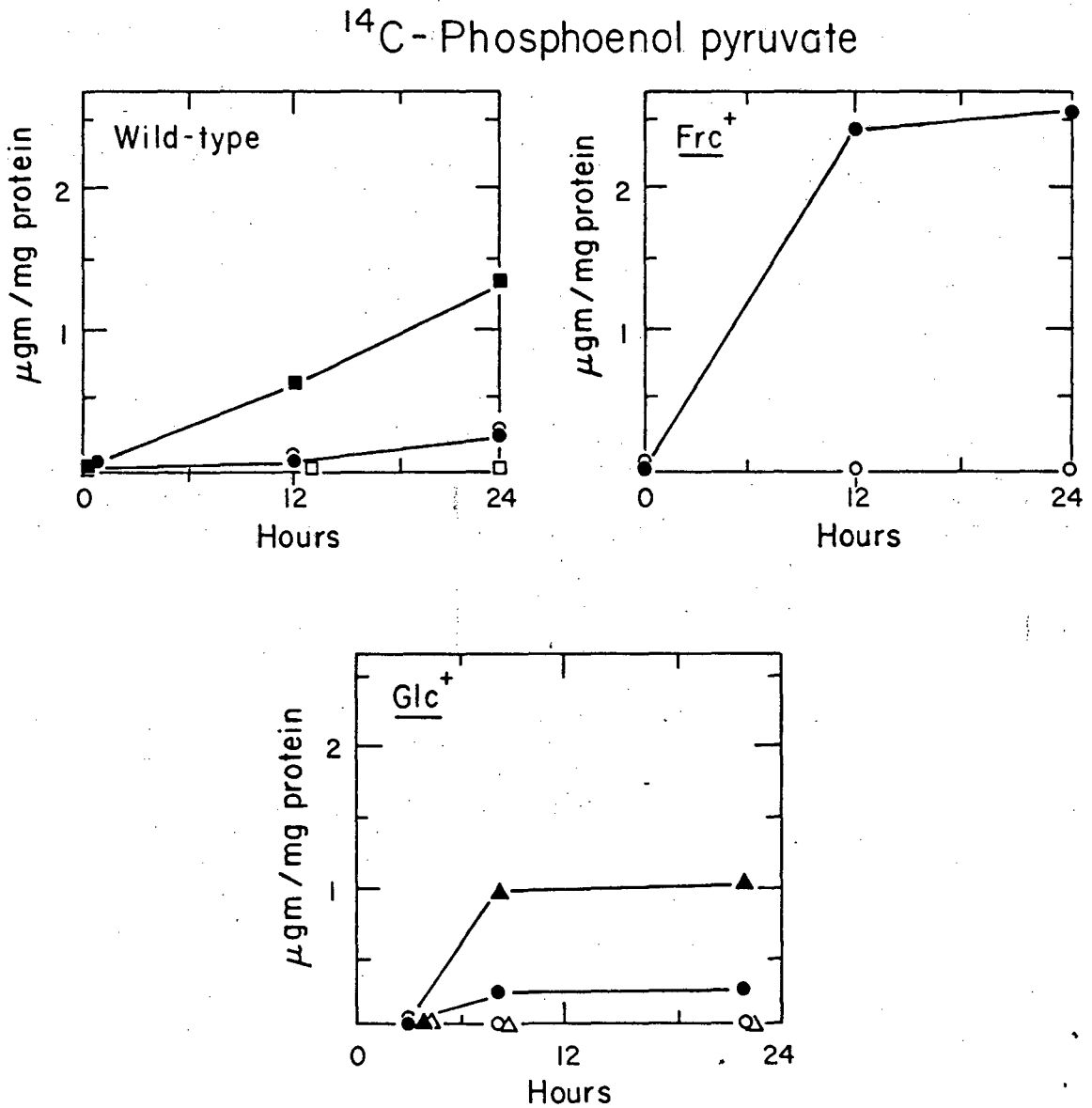

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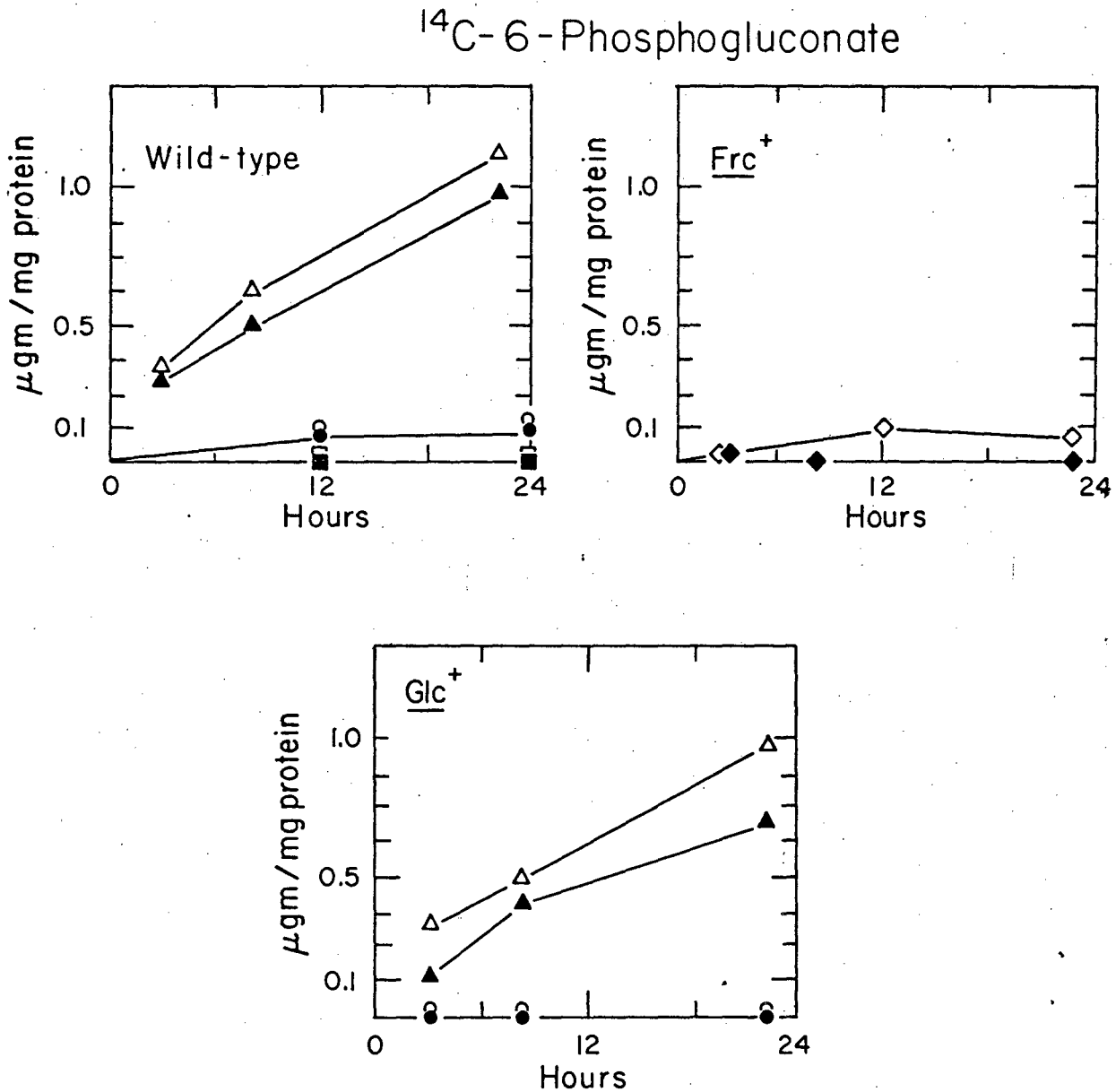

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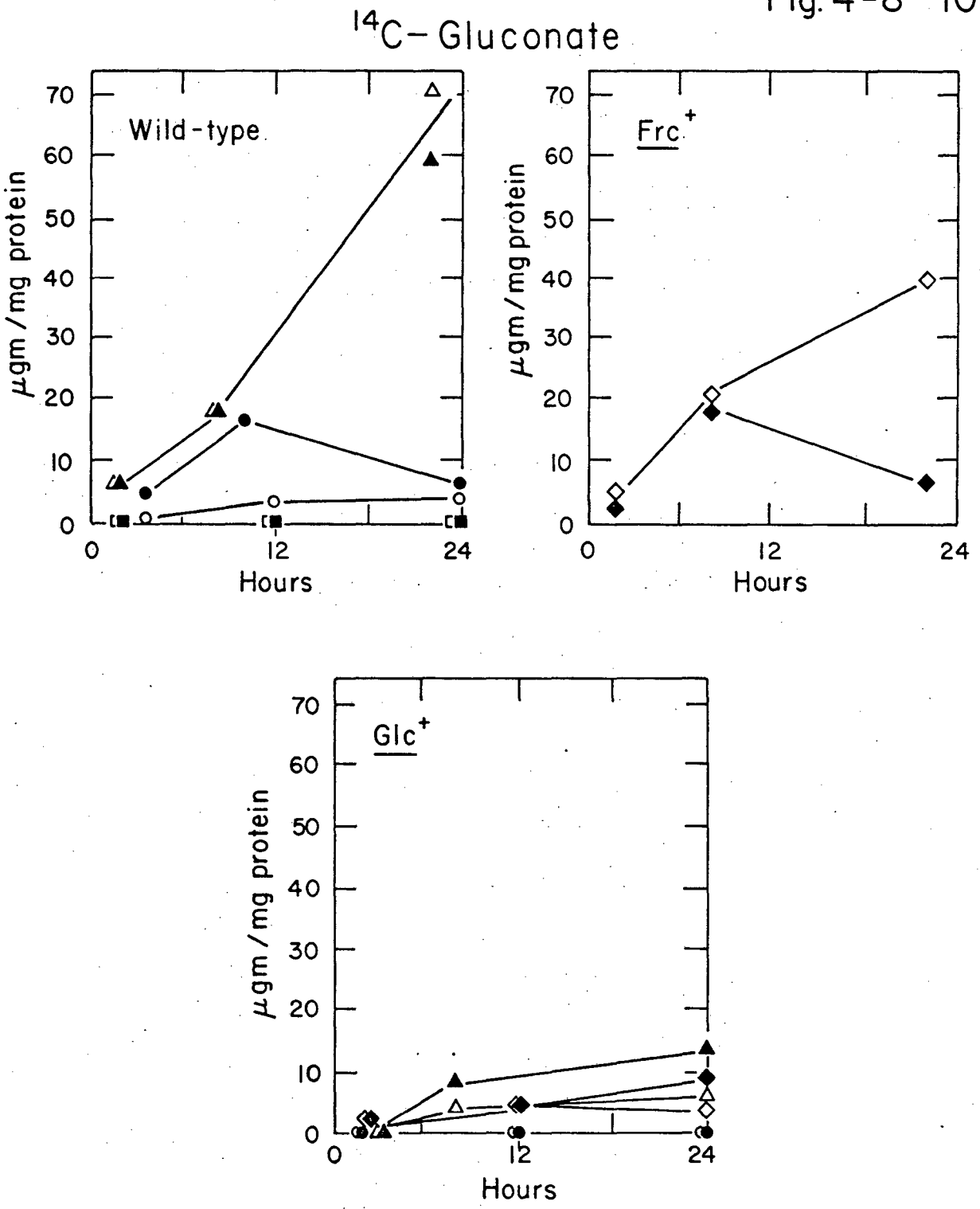

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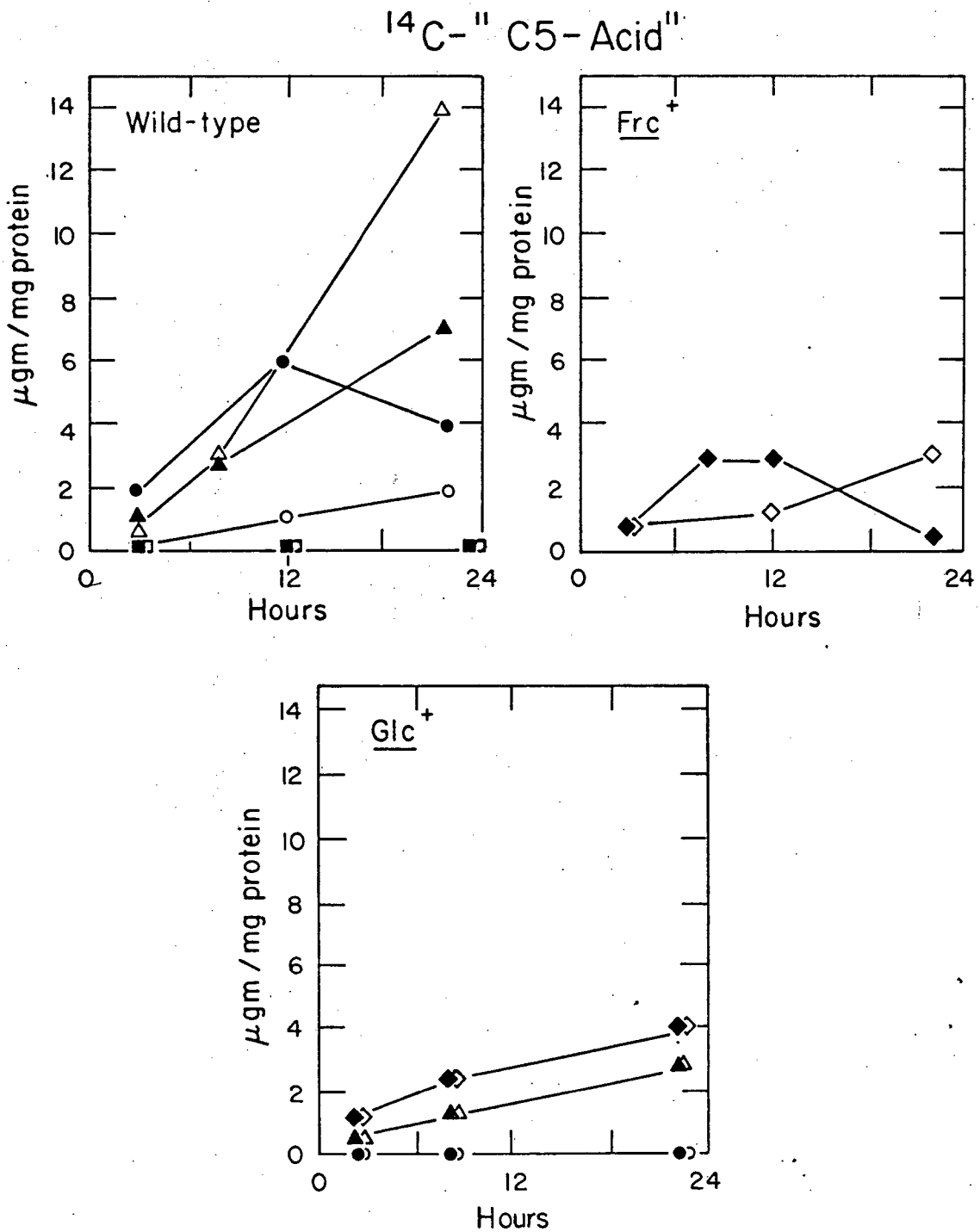

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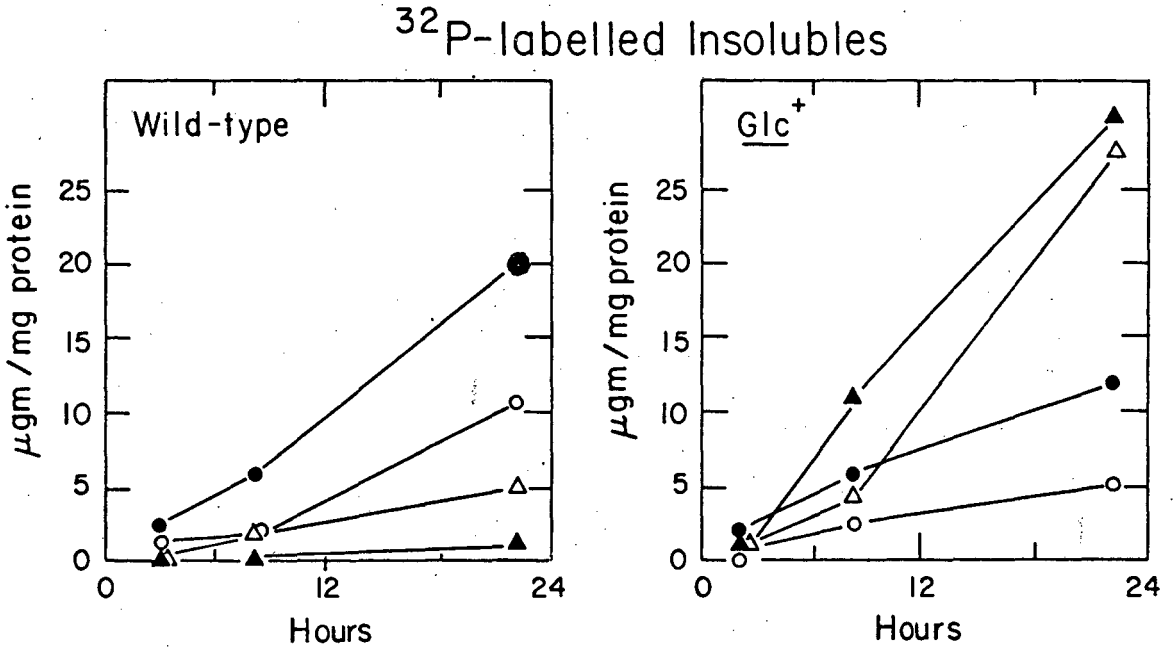

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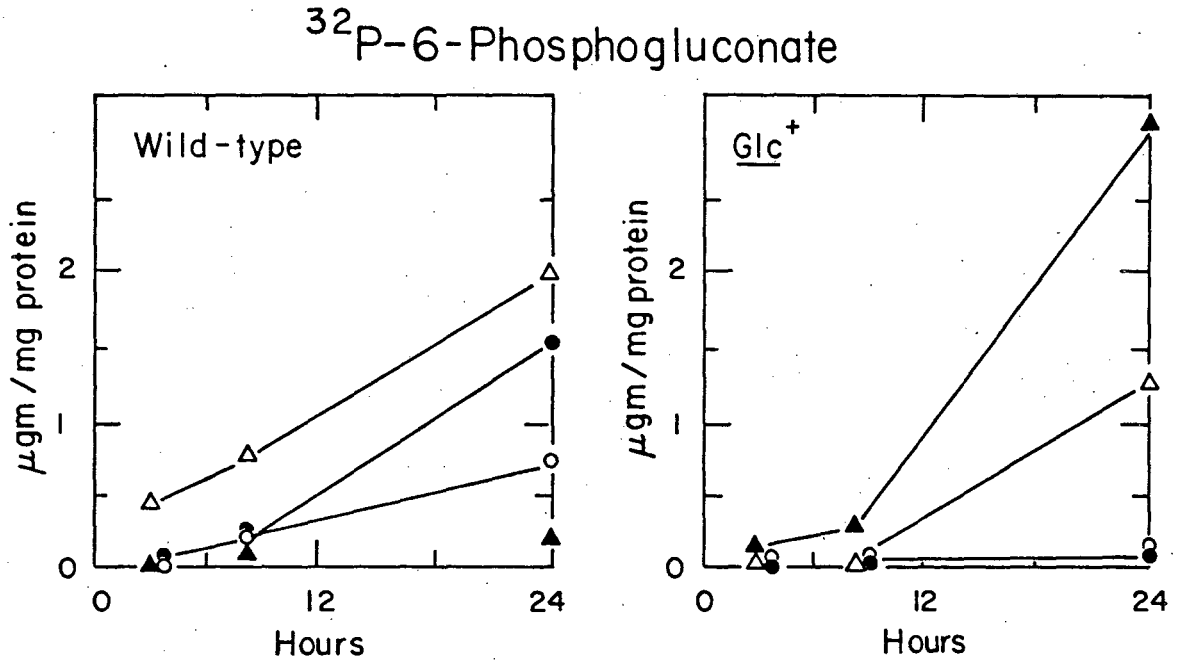

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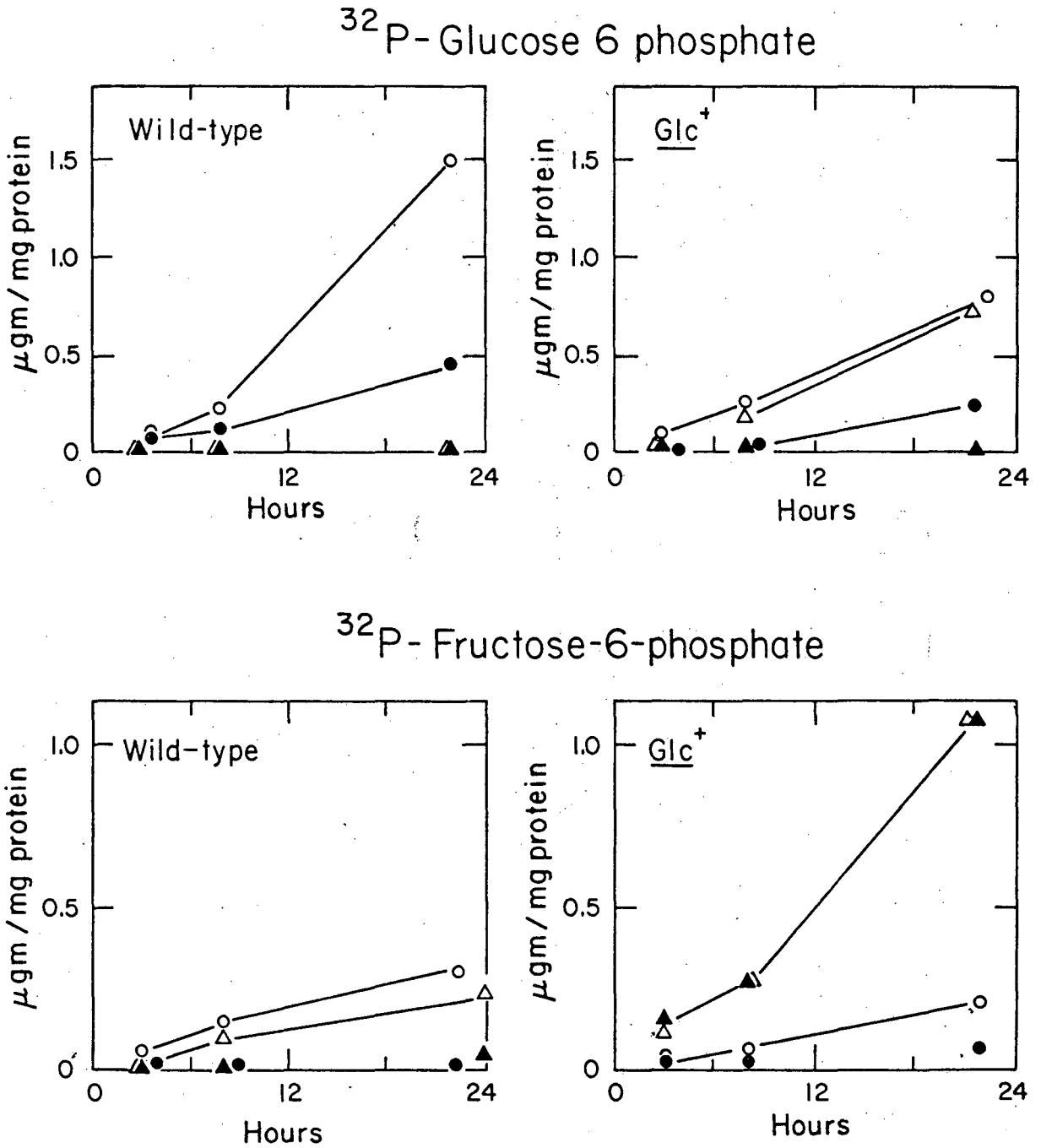

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Fig. 4-12 114

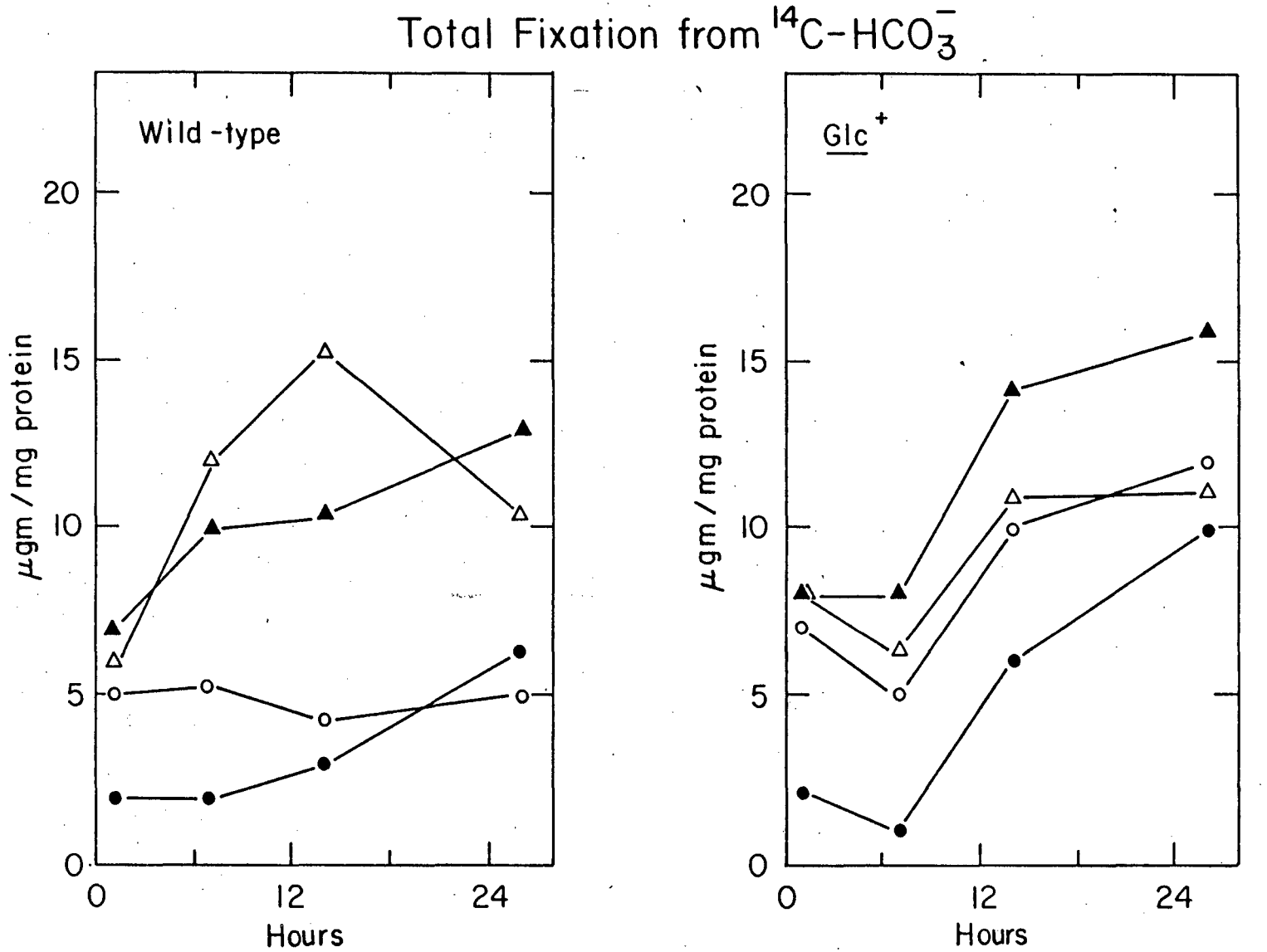
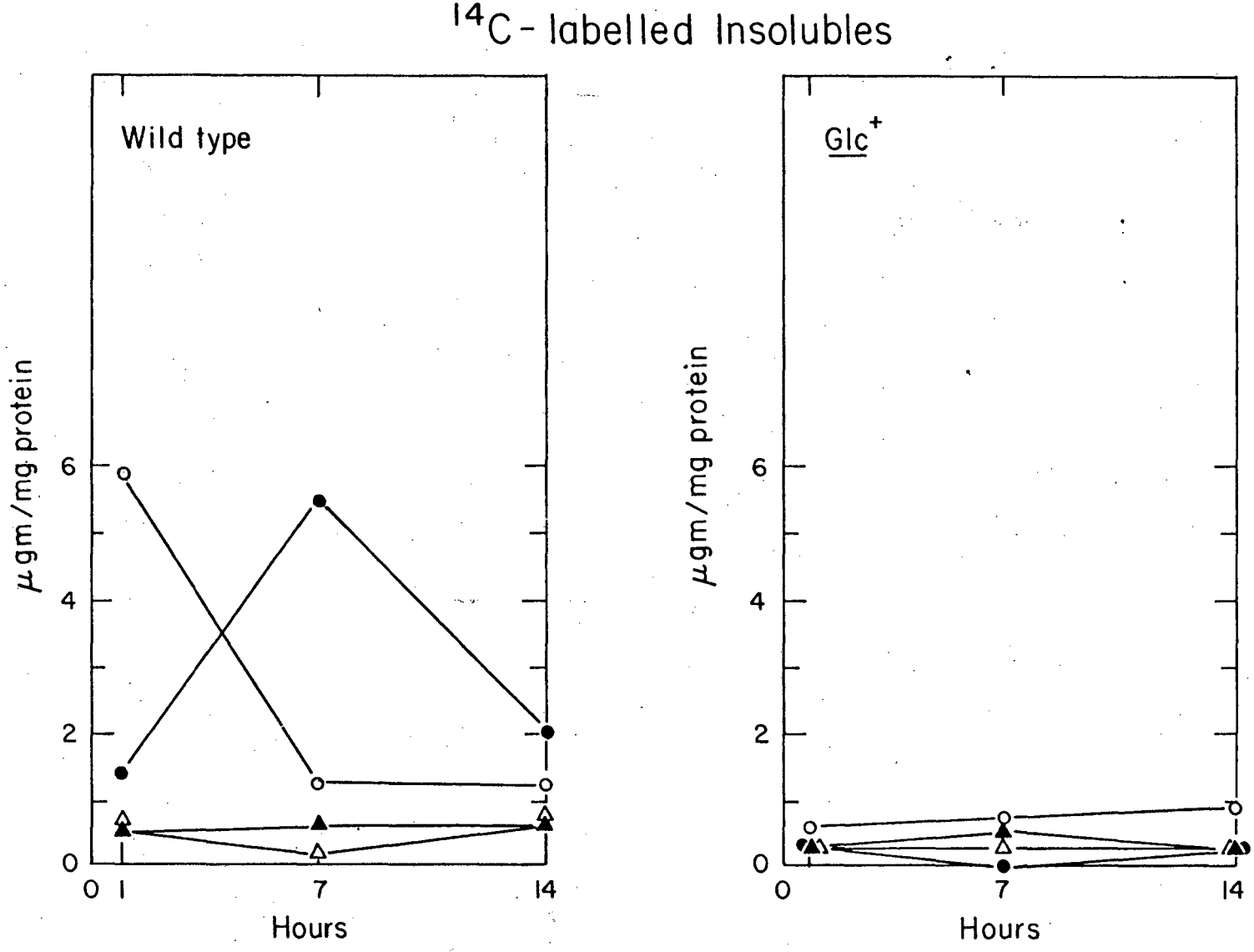

Total Fixation from $^{14}C-HCO_3^-$
Wild-type
Glc+
μgm / mg protein
Hours
0
5
10
15
20
0
12
24
XBL 8012-2636

Fig. 4-13



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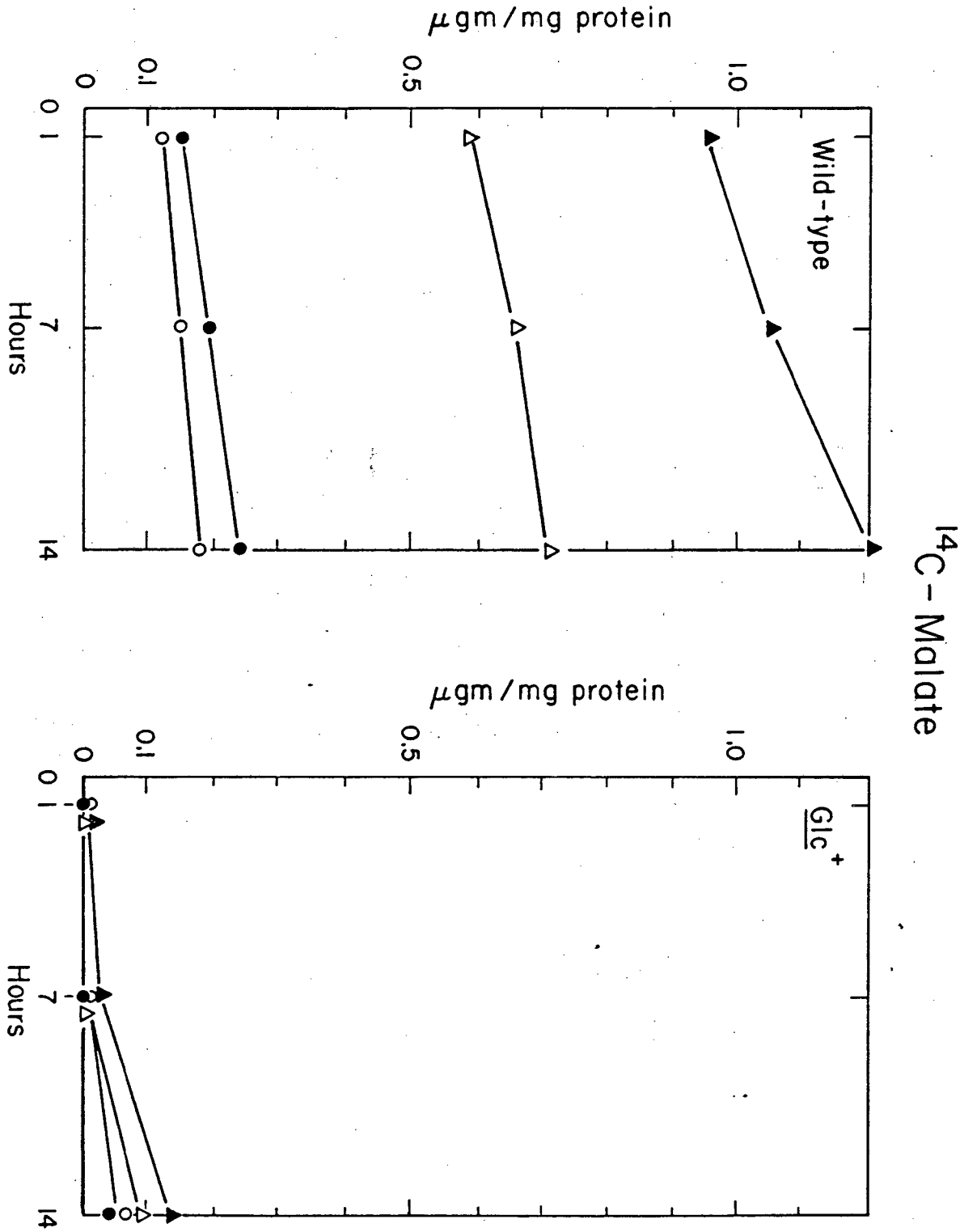

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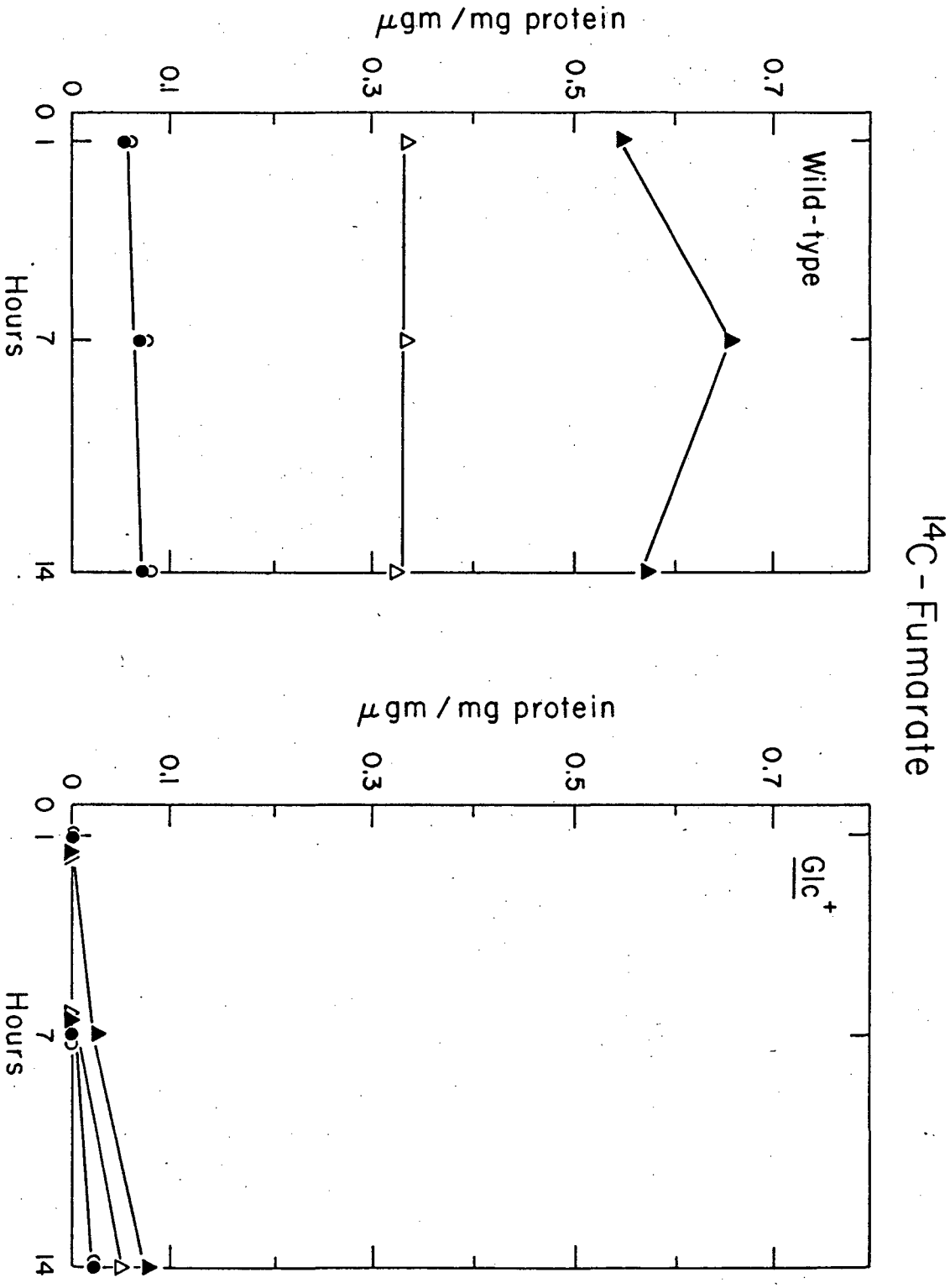

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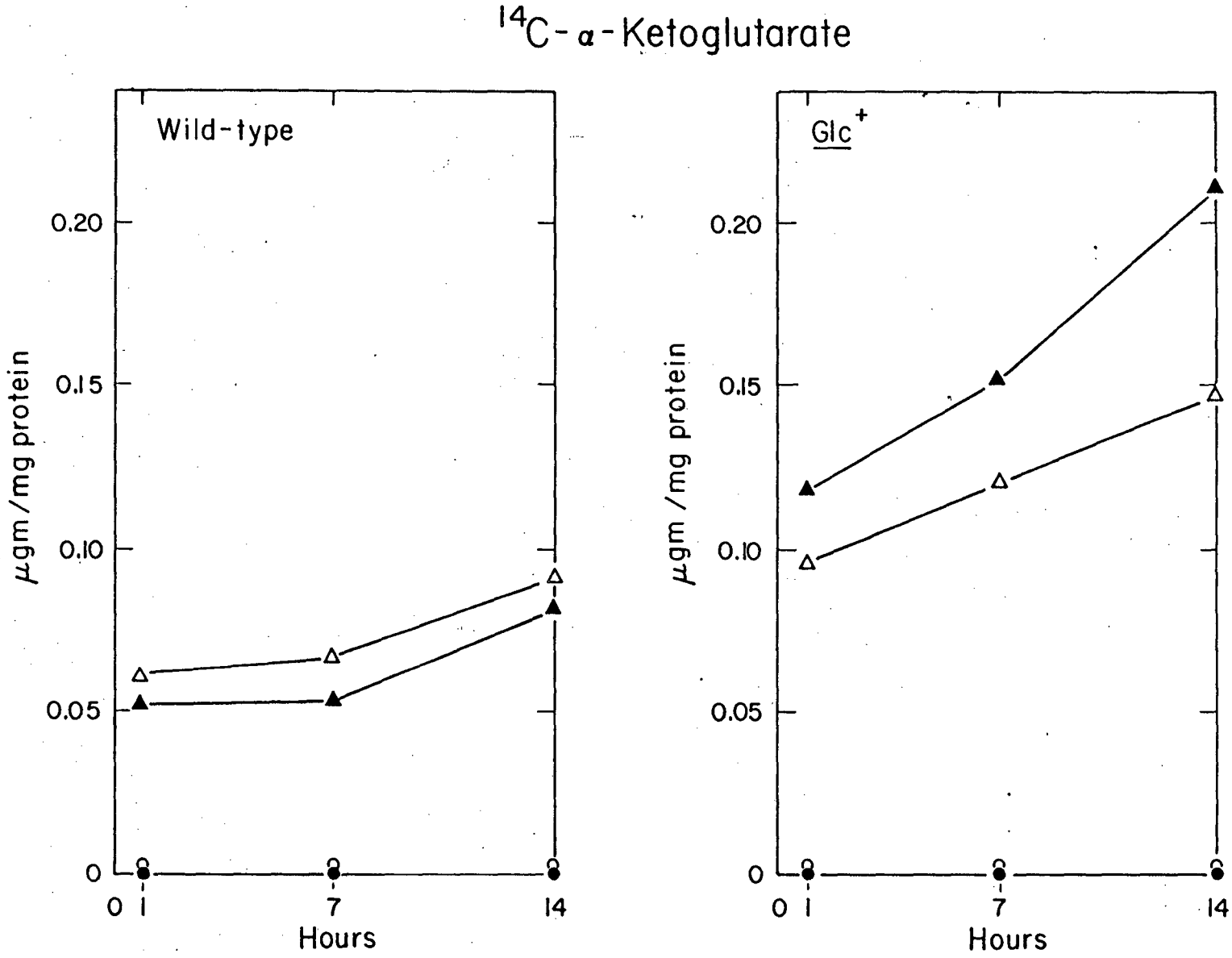
Fig. 4-16 118



XBL 8012-2634

Fig. 4-17

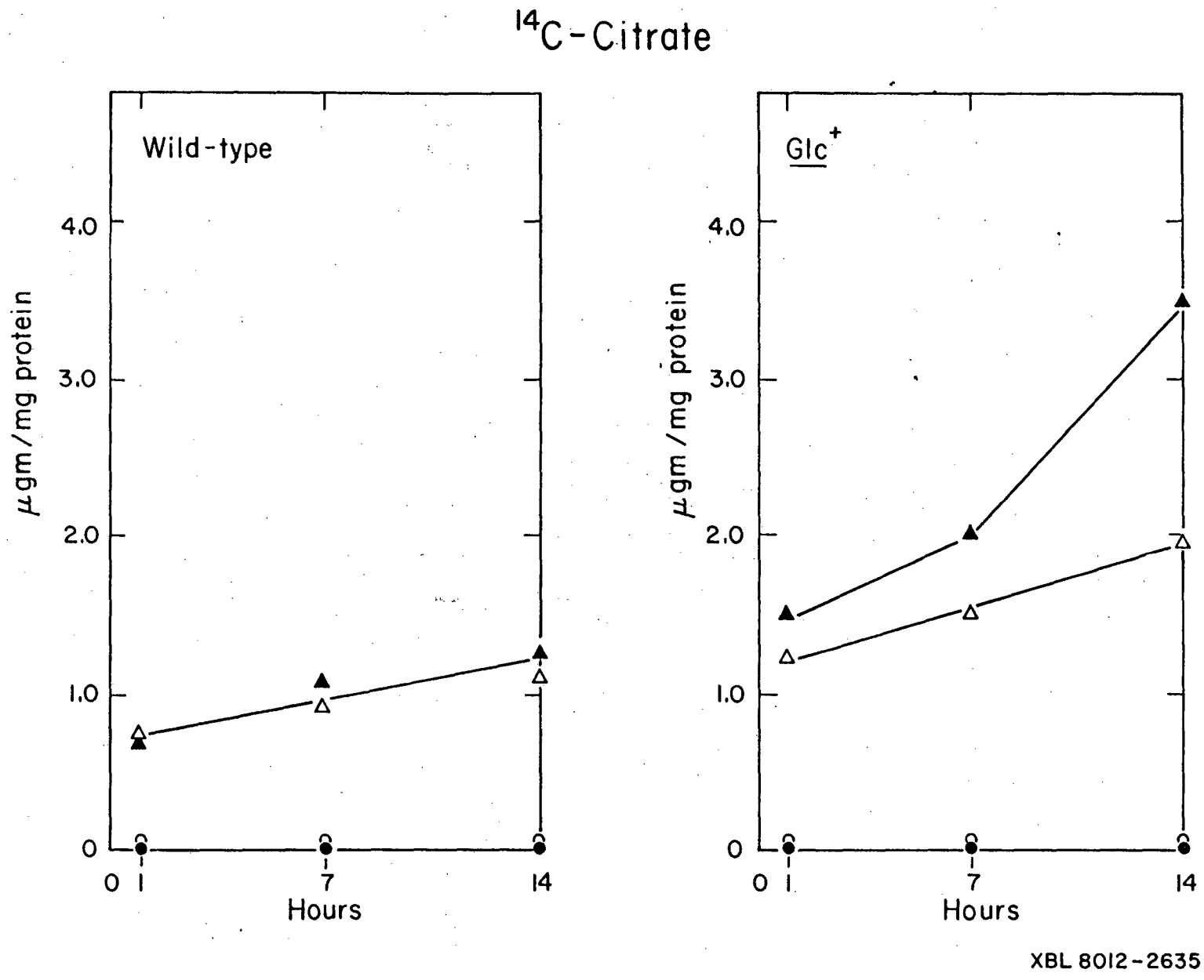

^{14}C-Citrate
Wild-type
Glc$^+$
μgm/mg protein
4.0
3.0
2.0
1.0
0
0
1
7
14
Hours
XBL 8012-2635

Fig. 4-18 120

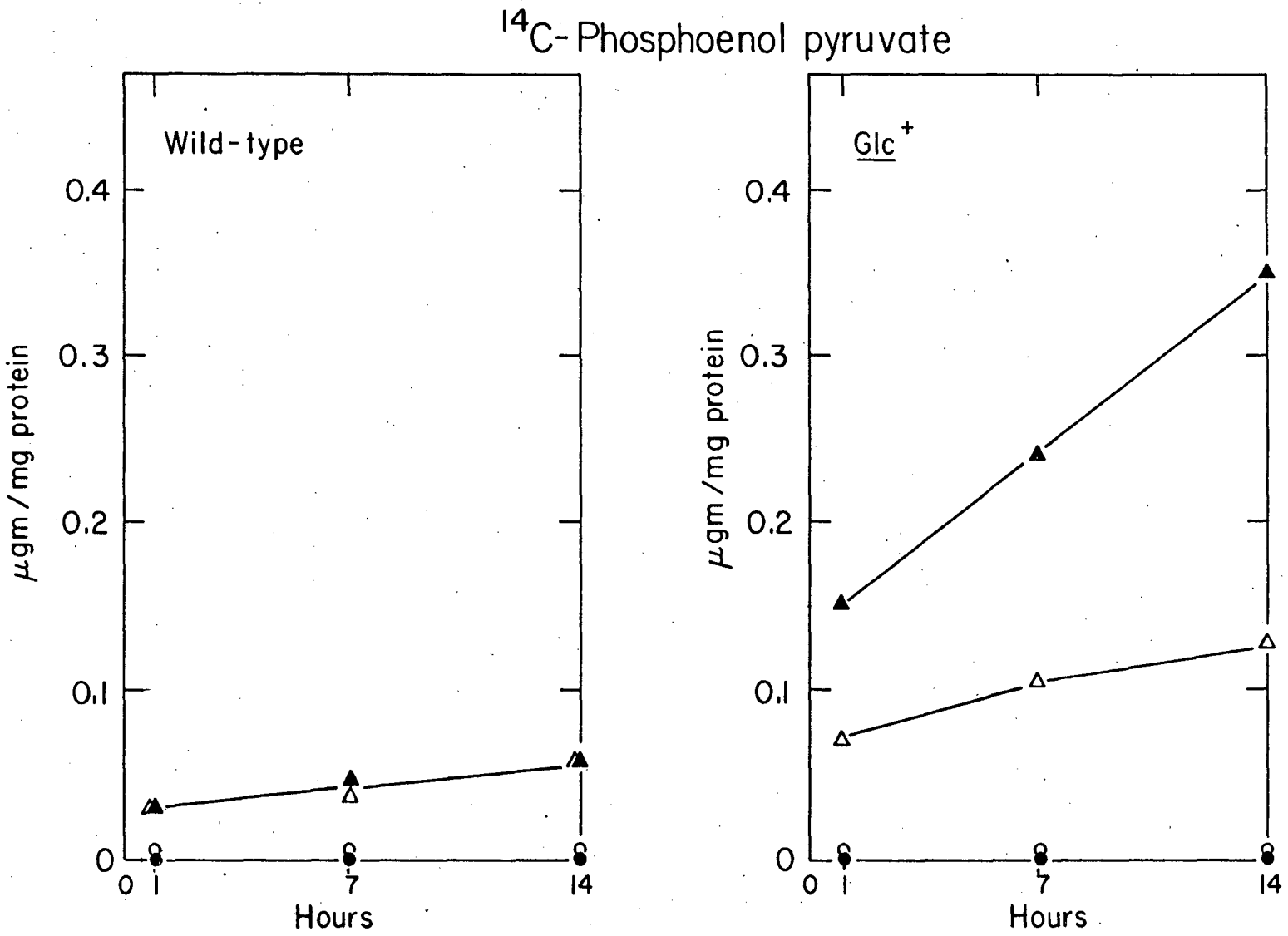
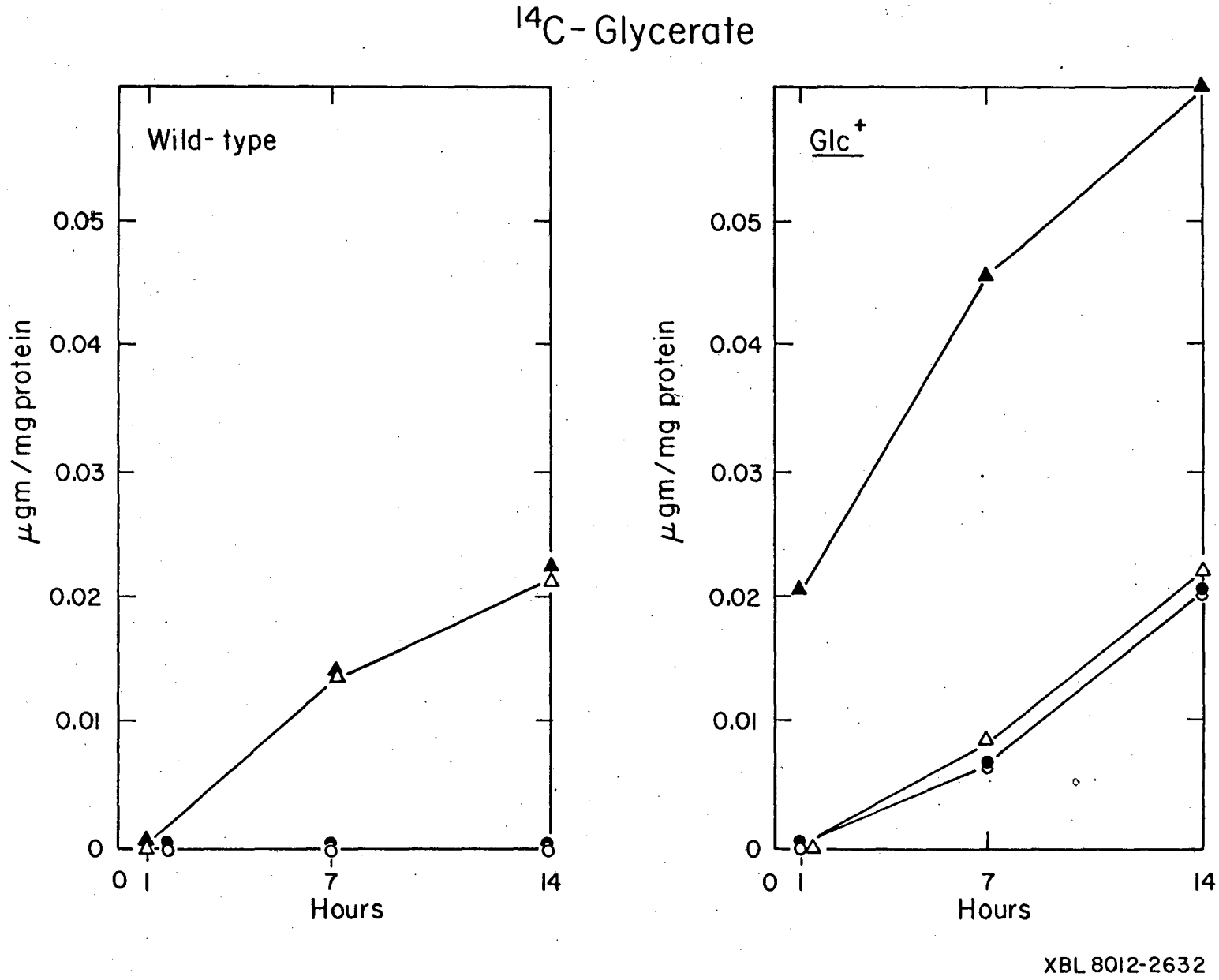
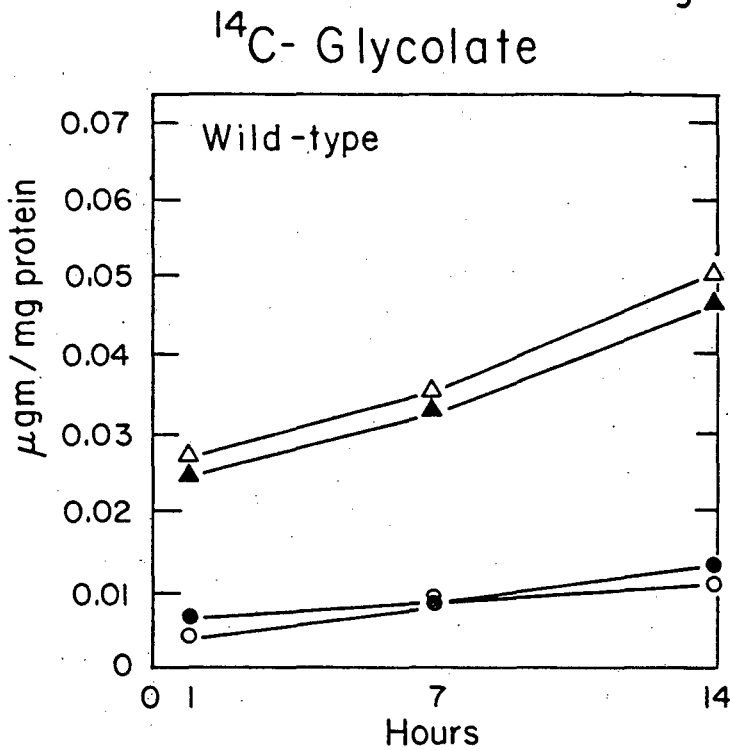
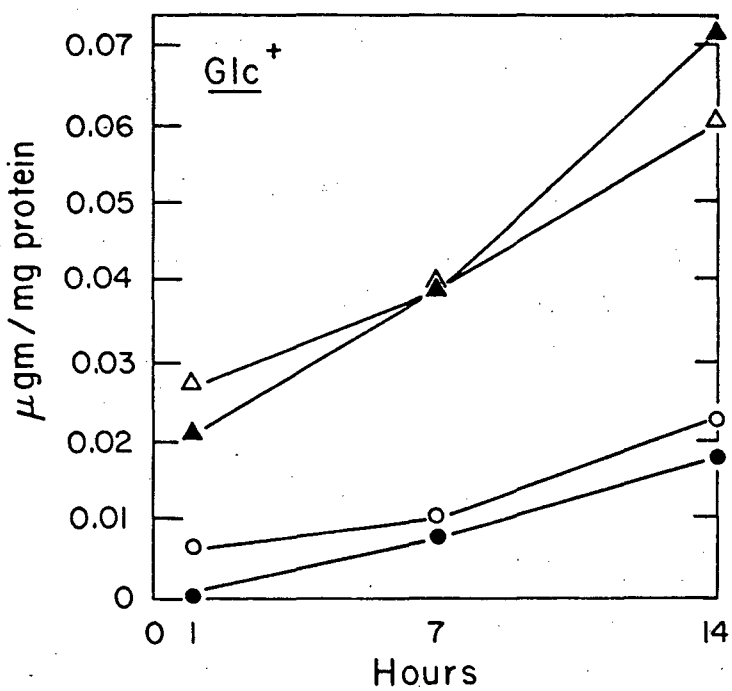

^{14}C-Phosphoenol pyruvate
Wild-type
Glc$^+$
μgm/mg protein
0.4
0.3
0.2
0.1
0
0
1
7
14
Hours
XBL 8012-2641

Fig. 4-19

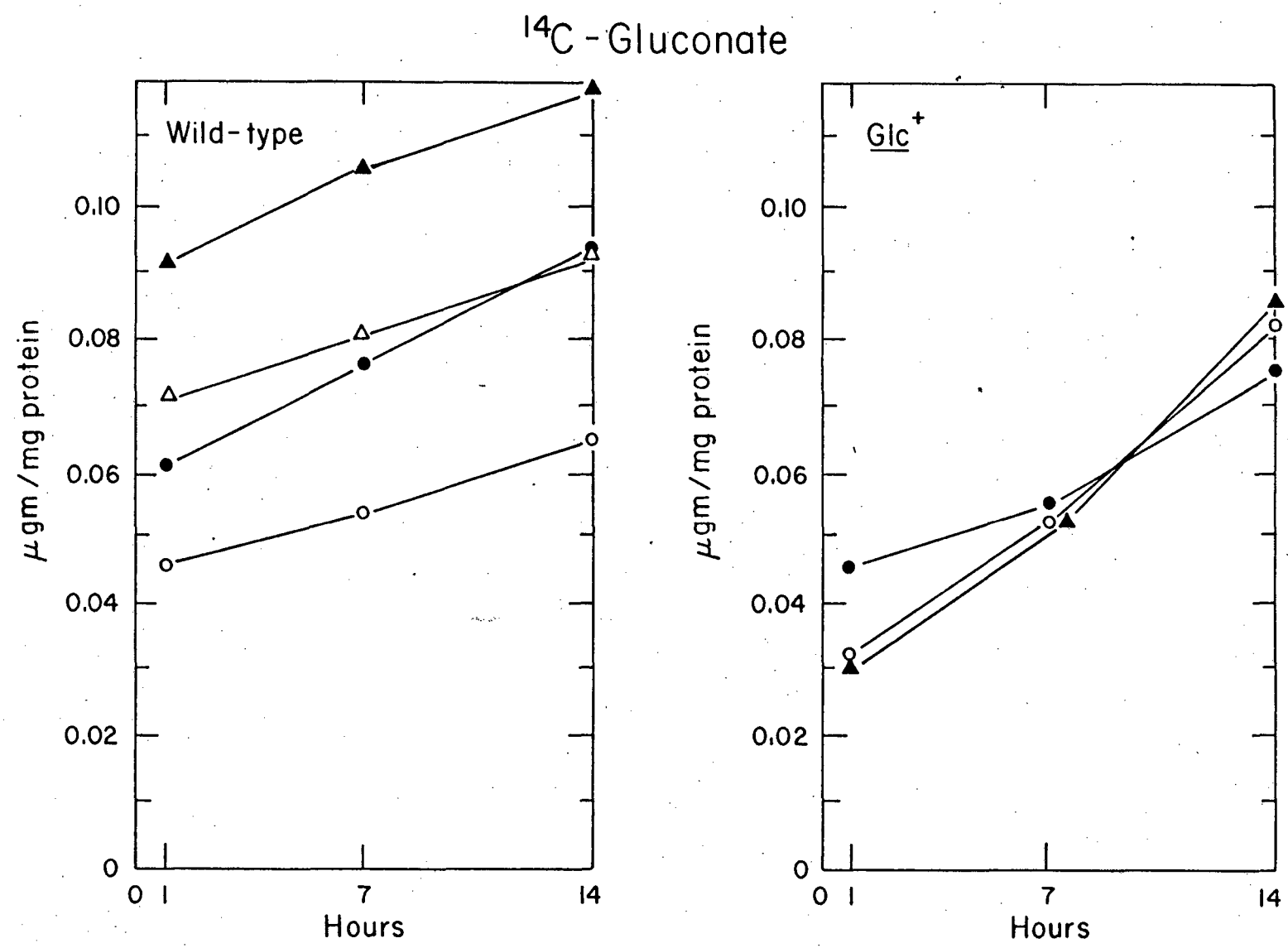

^{14}C-Glycerate
Wild-type
Glc$^+$
μgm/mg protein
0.05
0.04
0.03
0.02
0.01
0
0 1 7 14
Hours
XBL 8012-2632



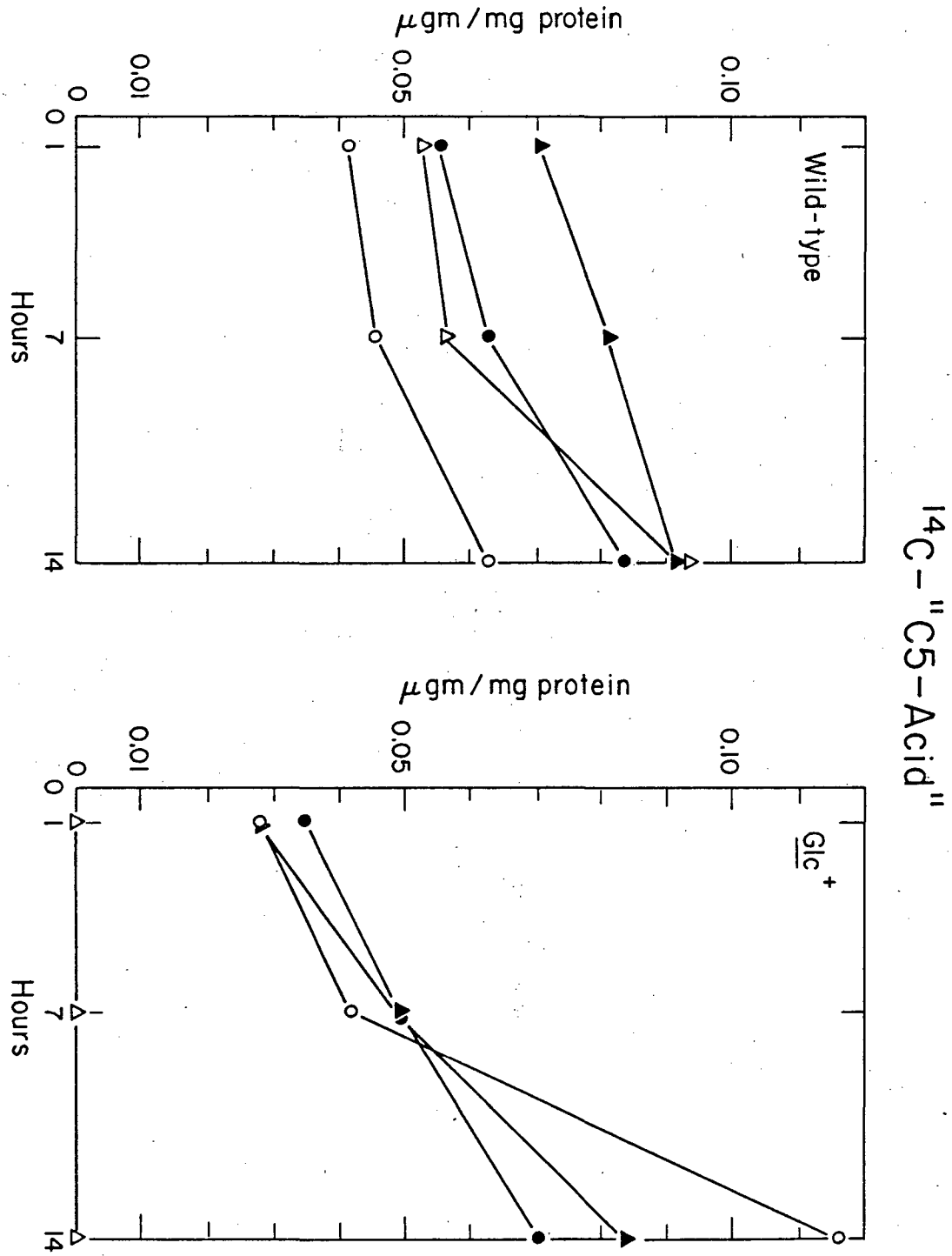


XBL 8012-2643

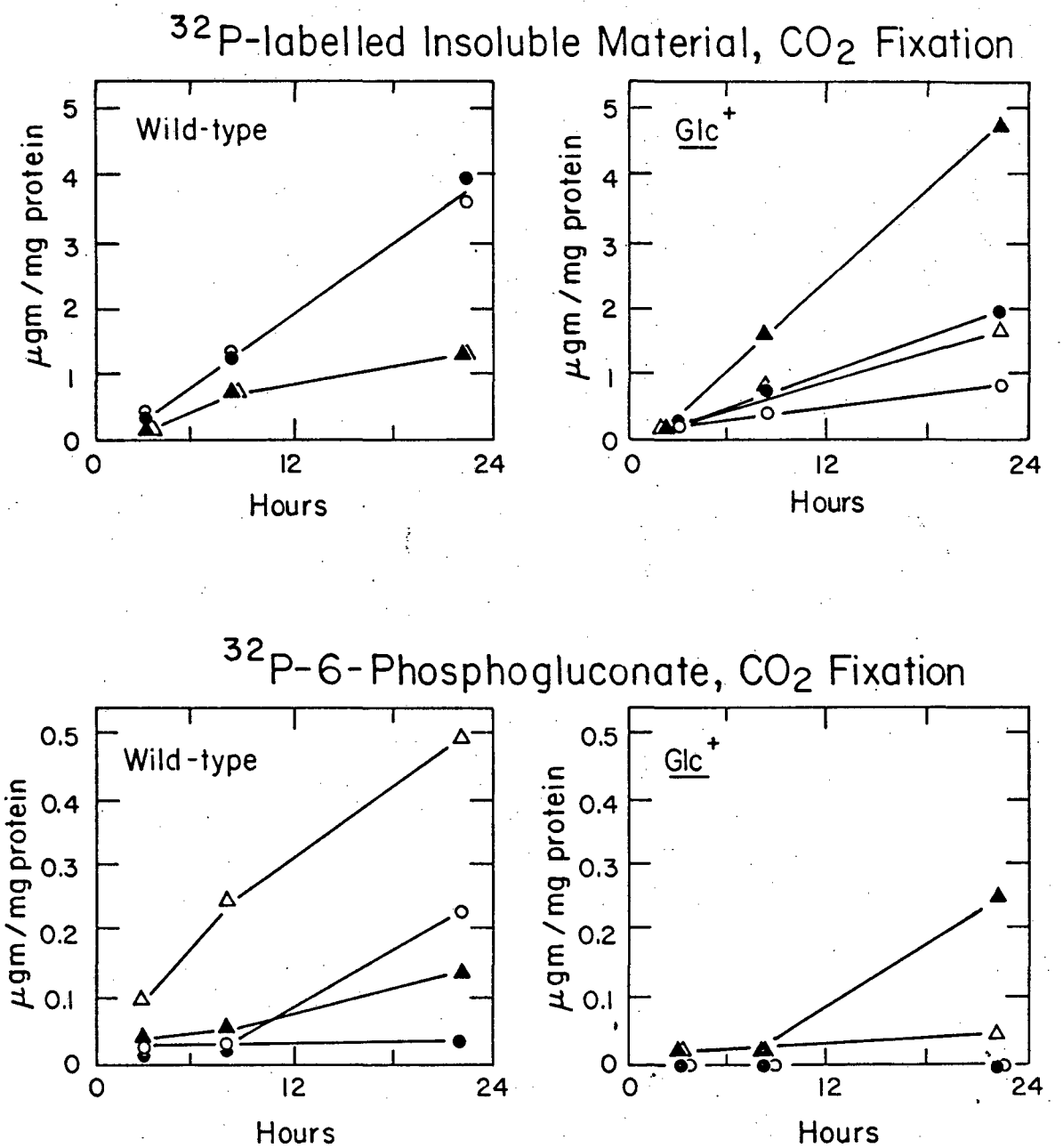
Fig. 4-21 123



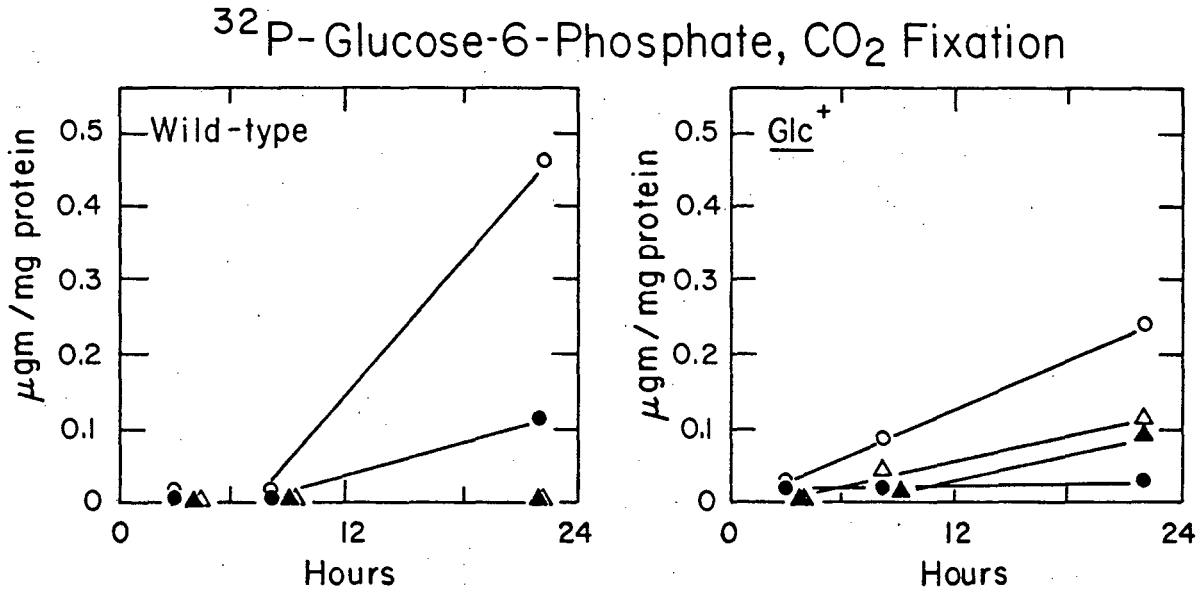
XBL 8012-2640

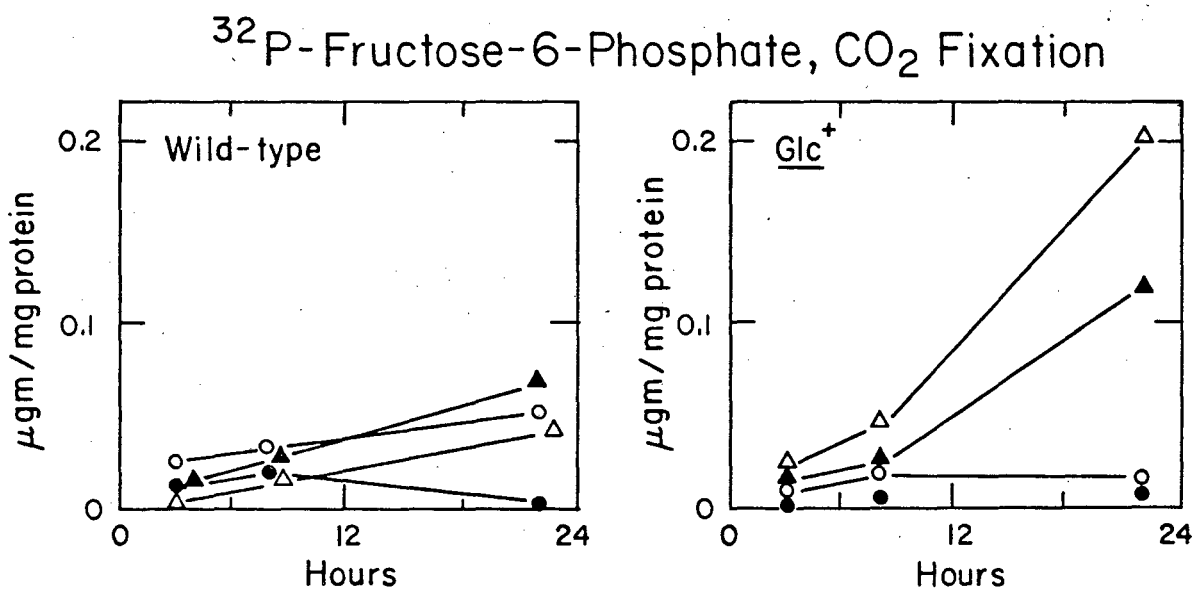


XBL 8012-2638A

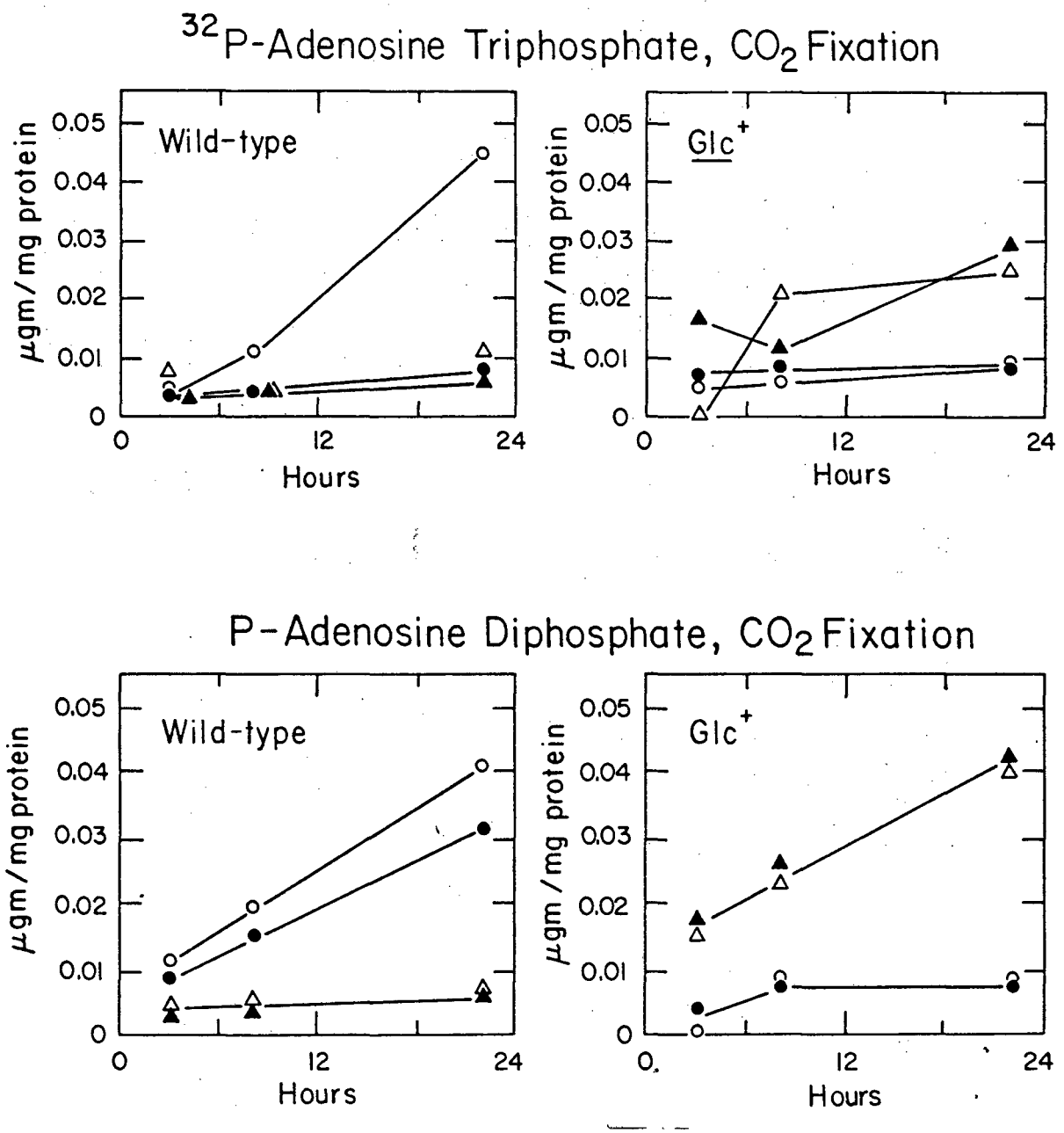


XBL 8012-2667

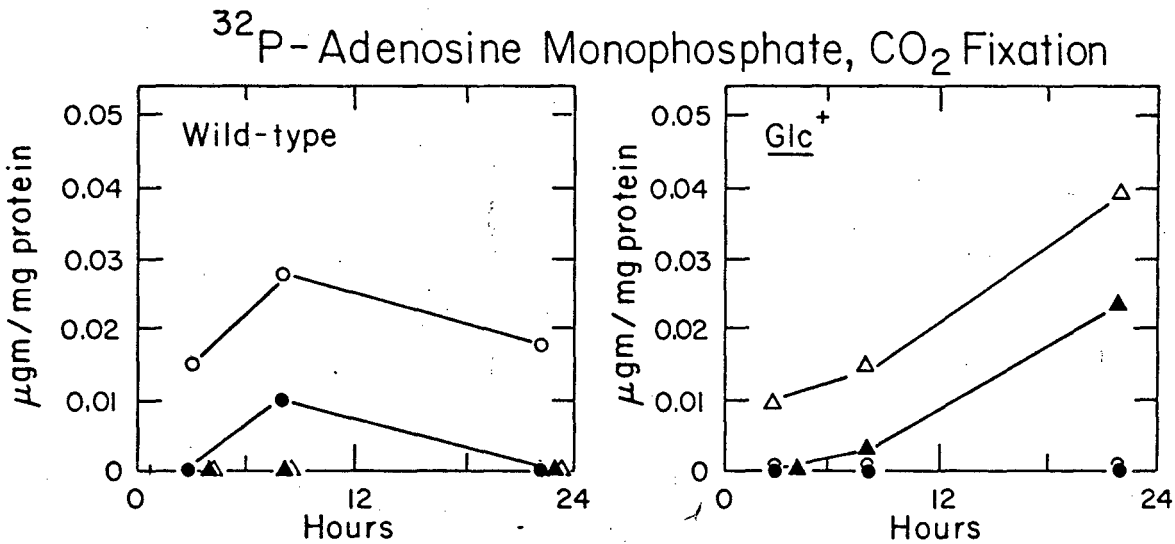




XBL 8012-2668



XBL8012-2669



XBL 8012-2670

CONCLUSION

The motivation for this research was to look at the usefulness and adaptability of R. sphaeroides as an organism capable of photosynthetic energy production. R. sphaeroides produces H_2 from reduced carbon sources with highest recovery when provided with light and specific culture conditions. These conditions include minimal ammonium ion and an anaerobic atmosphere. Efficiency of H_2 production in terms of recovery of reducing power in the wild-type is from 0% to about 50% depending on the carbon source provided. Energy for the conversion of substrate to H_2 is provided by light, although no net storage of this energy occurs.

It proved possible to isolate strains of this organism capable of growing on and converting to H_2 reduced carbon substrates that were not so utilizable to the wild-type. It was possible in this way to increase the efficiency of H_2 production to 50-95%, depending on the substrate. It appears as if this organism could be manipulated to develop strains capable of utilizing a wide variety of reduced organic compounds for H_2 production.

It was observed that reduced nitrogen in the form of ammonium ion significantly inhibited H_2 production. This would presumably be from its effect on nitrogenase, the enzyme responsible for H_2 biosynthesis in this organism, and on glutamine synthetase, the regulatory enzyme that controls the activity of nitrogenase. It has been reported recently (81,83) that strains of R. capsulata have been isolated that are deficient in glutamine synthetase. These show the ability to produce H_2 even when ammonium ion is in excess.

Reduced nitrogen in the form of amino acids seem to inhibit H_2 production little, if at all. H_2 was actually produced in cultures growing on hydrolyzed yeast. Cultures with glutamate as nitrogen source invariably produced H_2 before the glutamate was depleted. This suggests that a large-scale system that utilized non-specific wastes would have the potential for H_2 production even with significant amounts of fixed nitrogen.

It is not clear why this organism does not use the nitrogen and reduced carbon to grow. It might be that some other material is limiting. However, additions of trace minerals, vitamins and phosphate do not seem to stimulate growth or inhibit H_2 production. Rather, they seem to prolong it. Light does not appear to be limiting, either. Light provides the energy for cyclic photophosphorylation to produce ATP. Nitrogenase uses at least four molecules of ATP for each molecule of H_2 produced. Since H_2 production is extensive, ATP production must likewise be extensive. The rapid ^{32}P-phosphate labeling of the adenylates supports this.

The reuptake of H_2 and the fixation of CO_2, both of which would be expected to lower the absolute yield of H_2, seem to be relatively insignificant. The addition of carbon monoxide, an inhibitor of the hydrogenase that would use H_2, to H_2 producing cultures showed little or no effect on H_2 production. CO_2 fixation was observed to be $\leq 1\%$ of the CO_2 released in substrate metabolism. The removal of CO_2 from the cultures by trapping or sparging did not appear to quantitatively change H_2 yields.

Cultures of R. sphaeroides were capable of H_2 production when resupplied with reduced carbon substrates for periods of at least a month. H_2 was produced in continuous cultures of volumes of 10-20 l. There is no reason to believe that larger cultures would not also produce H_2.

It appears then, that R. sphaeroides is an excellent organism for light-mediated energy production utilizing reduced organic wastes.

It remains to speculate on the role of H_2 production in R. sphaeroides. It is associated primarily with the overabundance of reduced carbon substrate, a high light intensity, an anaerobic environment and the absence of enzymatic and metabolic inhibitors. Growth during H_2 production is minimal. This metabolism comes at the expense of flow into storage materials. What it accomplishes is the depletion of substrate and the maintenance of an anaerobic environment. The supposed function of nitrogenase to the cell is the fixation of N_2 into ammonia, which is incorporated into glutamine. This would help satisfy the cell's need for fixed nitrogen. The presence of ammonium ion in the system reroutes carbon flow dramatically, with an inhibition of the nitrogenase system and a stimulation of incorporation of the carbon substrate into cellular materials. When N_2 is low or absent from the atmosphere, H_2 is produced. A working hypothesis over the years has been that this function is a regulatory one that allows the cell to balance energy conversion and biosynthesis. When excess reducing power or energy charge is present without the materials for cell growth, the cell dumps energy and electrons into H_2 production. It has recently been reported that R. capsulata will produce H_2 low light intensities, where the cells might be limited for energy charge (37). In this case, H_2 production would actually be a bioenergetic burden. My own studies have shown that the energy charge and $(NAD(P)H)/(NAD(P)^+)$ ratios are low during H_2 production with R. sphaeroides. Thus the significance of H_2 production to the cell may not be always energy balance.

In assessing my contribution to the field, I see that much of my work with *R. sphaeroides* is confirmation of work performed previously with other closely related species. This was particularly true of my initial work on the phsyiology and enzymology of H_2 production.

The *in vivo* measurement of energy charge and reducing potential during H_2 production was novel. The development and characterization of H_2 producing substrate mutant strains was not unique, although the discovery that the wild-type and Glc^+ mutant both metabolized glucose via the Embden-Meyerhof pathway was unexpected and significant to the field. The Entner-Doudoroff pathway cannot be considered the principal route of hexose metabolism, as was held to be true previously. *In vivo* labeling of bicarbonate fixation during H_2 production has never been done before and the demonstration of fixation solely by phosphoenolpyruvate carboxylase under these conditions will force a reevaluation of photosynthesis during photoheterotrophic culturing.

It is clear that we cannot hold photosynthesis, photophosphorylation, carbon metabolism and N_2 fixation to be regulated separately as entire units when they occur simultaneously in a living cell. This work, being exclusively *in vivo* in nature, points out again that the organism uses its environment to determine what it will do and what paths it will take.

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