Submitted to the Journal of Bacteriology

LBL-8227
Preprint

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September 1978

Prepared for the U. S. Department of Energy
under Contract W-7405-ENG-48

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QUANTITATIVE HYDROGEN BIOSYNTHESIS FROM A RPS. SPHAEROIDES MUTANT

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Running head: Hydrogen Biosynthesis in Rps. Sphaeroides

ABSTRACT

Rhodopseudomonas sphaeroides produces molecular H_2 and CO_2 from reduced organic compounds which serve as electron sources and from light which provides energy in the form of ATP. This process is mediated by a nitrogenase enzyme. A mutant has been found that, unlike the wild-type, will quantitatively convert glucose to H_2 and CO_2. Techniques for isolating other strains capable of utilizing other unusual electron sources are presented.

Metabolism of glucose by the wild-type strain leads to an accumulation of gluconate. The isolated mutant strain does not appear to accumulate gluconate.

The purple, non-sulfur bacteria have long been known to produce molecular hydrogen under defined conditions (5). Although low rates of hydrogen biosynthesis have been reported for dark-grown cultures of these bacteria (6), the light-mediated process appears to be quantitatively more significant and has therefore attracted more study. These organisms utilize reduced carbon compounds and water as electron donors. For those compounds studied, the process yields CO_2 and H_2 as the sole products of conversion (7). Equations (1) and (2) show the stoichiometry of this photoconversion process for glucose and malate:

$$(1)\quad C_6H_{12}O_6 + 6\ H_2O \xrightleftharpoons{\text{light}} 12\ H_2 + 6\ CO_2$$

$$(2)\quad C_4H_4O_5 + 3\ H_2O \xrightleftharpoons{\text{light}} 6\ H_2O + 4\ CO_2$$

Depending on the organic substrate, there may or may not be any net storage of chemical energy, but the energy to drive the conversion at a significant rate comes from light, presumably via energy stored in ATP formed by photophosphorylation, by photoelectron transport, or both. Although the standard degradative pathways have been demonstrated for these organisms (1), the specific pathways responsible for hydrogen production have not been identified.

Historically, hydrogen biosynthesis has been considered to be mediated by nitrogenase and hydrogenase enzymes. Hydrogenases as a class are reversibly inhibited by oxygen, which appears to compete with protons as an electron acceptor in the reductive process. They are non-competitively inhibited by carbon monoxide, which may affect the obligatory electron-transport chain. Cytochrome C_3 is required for activity (18). ATP is not required for enzyme activity, but could be required for metabolism leading to reduction of electron carriers to reductants of sufficient electro-negativity.

Nitrogenases are irreversibly inhibited by oxygen. They are non-competitively inhibited by CO, which may affect the transfer of electrons

from the Fe subunit to the Mo-Fe subunit. Curiously, while all substrate reductions occur on the Mo-Fe subunit, proton reduction (hydrogen production) is unaffected by CO (9). At least two ATP molecules per electron transferred are required for the enzymatic reaction (14,18).

An understanding of H_2 biosynthesis by non-sulfur purple bacteria requires knowledge of the extent to which these two systems operate in the cell. Hillmer and Gest (8) have discussed the problem and concluded that nitrogenase appears to be the sole enzyme responsible for the H_2 biosynthesis in Rps. capsulata. We present here similar results for Rps. sphaeroides.

A long range goal of this research is 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 hydrogen production would require bacterial strains capable of utilizing the various kinds of reduced compounds that might be readily available, such as agricultural, paper mill or sewage organic wastes. In this paper we demonstrate one such strain of Rps. sphaeroides capable of quantitatively converting glucose to CO_2 and H_2 when in the light. Some properties of this strain and how other strains may be isolated for other substrates are briefly described.

MATERIALS AND METHODS

Organisms. Wild-type Rhodopseudomonas sphaerodies was obtained from the Berkeley Collection. Strains capable of producing H_2 from unusual carbon sources were isolated as described in Results.

Media and culture conditions. Cells were grown and maintained in 15 ml screw cap vials in a modified Hutner's medium of 2% (v/v) Hutner's concentrated mineral base with growth factors (2), 1% (w/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 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. (13).

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 NaOH gas trap (Fig. 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 quantified 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. 2). Flasks contained 50 ml minimal medium and a gas phase of known volume, 50-70 ml. The trap contained 0.1NaOH. 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 H_2-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 innoculated with 1-5% by volume of a stationary culture, and the flasks were assembled and flushed with the appropriate atomsphere. 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 quickly 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 5 Å molecular sieve, with peaks integrated with a Hewlett-Packard 18652AA/D converter or using a Hewlett-Packard 5830A gas-liquid chromatograph with a column of carbosieve 101. Carrier gasses were helium and nitrogen, respectively. It was found that the alkaline traps of system (i) and (iI) removed 85-90% of the atmospheric CO_2.

Nitrogenase assay. Nitrogenase activity was estimated by acetylene reduction (11). Cultures in various growth states in system (ii) flasks were flushed with 10% acetylene in argon for about five minutes which was long enough to be complete as shown by gas-liquid chromatographic (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 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 withdrawn directly from above the nitrogen-limited cultures as described above.

^{14}C tracer experiment. 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 900 lux illumination. As gas production began (10-20 hrs), low levels (0.4-0.8 mC/mmole) 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 atmospheric CO_2. After gas evolution ceased, the culture media was collected and placed inside wells of Warburg glasks containing 2N 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 Gieiger-counting (10,16). The solvent system for the first dimension was phenol-water-glacial acetic acid-ethylenediamine tetraaceitc acid (M) (840:160:10:1 v/v/v/v)

and in the second dimension, equal volumes of n-butanol-water (370:25 v/v) and proprionic 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. Uniformly labelled 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 per 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 0.4 ml of an organic base (NCS Tissue Solubilizer, Amersham/Searle) in the center well. One ml of 4 N HCl was then added to the main chamber of the vessel and the vessel quickly sealed with a rubber stopper. Acidification of the supernatant fractions converted the carbonate ions to CO_2 which was then trapped in the organic base in the center well. 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, and the center well washed several times with NCS-solubilizer. All of the washings and the original contents from the center well were then placed in a commercial scintillation fluid (Multisol, Isolab Inc.) and counted for carbon-14 with an IsoCap/300 6868 liquid scintillation system (Searle Analytic Inc.). The amount of quenching was estimated using an external standard method programmed for the scintillation counter.

The analysis of radioactive carbon-14 assimilation was carried out as follows: cells were washed 4x with the 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 colormetrically by Glucostat reagent (Worthington Biochemical Corp.) or by quantitative gas liquid chromatography of the trimethylsilyl (TMS) derivative. Gluconate and an unknown sugar or 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.(12). The FID detector response was linear from 0.01 to 0.1 micrograms of glucose or gluconate. All measurements were made at a constant temperature of 195°C with nitrogen 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 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 minus-30°C until analysis was carried out. GLC analysis was carried out by injection of from 0.5-2.0 µl of the silylation mixture directly onto the column of the gas chromatograph.

RESULTS

Isolation of permissive strains. To isolate strains of Rps. sphaeroides able to produce H_2 gas from glucose or gluconate from wild-type which do not

ordinarily produce H_2 gas, the wild-type cells were spread on 2% (w/v) agar plates made with rich medium and 10 mM glucose or gluconate. 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 and 10 mM carbon source in 25 ml tubes with traps. These tubes were placed in the light at 27°. Cultures corresponding to the tubes producing H_2 were retained. In this way, strains capable of producing H_2 when grown on glucose (glc +) or gluconate (gnt +) were isolated.

Metabolism of substrates. Wild-type Rps. sphaeroides utilizes malate and lactate efficiently, both in terms of maximum rate and in terms of total conversion (Fig. 3). Glucose produces little H_2 and only after a considerable lag. Gluconate produces no H_2. Both compounds support dense photosynthetic growth, however. Citrate supports no growth above controls on minimal medium alone and such cultures do not produce H_2. The glc + strain procedures H_2 when supplied with malate or lactate with wild-type efficiency and produces high levels of H_2 when supplied with glucose (Fig. 4). The gnt + strain also uses malate or lactate with efficiencies similar to the wild-type, but also can make appreciable amounts of H_2 when supplied with gluconate.

Cessation of H_2 production by 100 hr (Fig 3a, 4a) indicates only depletion of substrate. If a utilizable substrate is added as the H_2 production rates begin to decline, the rates will rapidly increase, often above the initial maximum (Fig. 5). Periodic replenishment of the culture medium with yeast extract or trace elements of the modified Hutner's mineral base also extended H_2-production up to at least six weeks, although the rate of gas evolution was reduced (Fig. 6).

Culture conditions vs. H_2 production. Ammonium ion concentration and light intensity were both important in determining rates of H_2-production by Rps. sphaeroides (Fig. 7-8). Comparatively high (20 mM) and low (0.5 mM) levels of NH_4Cl did not support or only poorly supported growth (7B) and did not support H_2-production (7C). The optimum initial NH_4Cl level both for growth of cells and for H_2-production was observed between 2-10 mM. However, the concentration of NH_4Cl fell to very low levels (i.e. $\leq$ 0.5 mM) before H_2-production was initiated (6A). H_2-production was strongly influenced by the intensity of the incandescent light source (Fig. 8). H_2-production increased at an approximately linear rate in the range of 100-1200 ft candle, although intensities greater than 4000 ft. candle inhibited the production of H_2 gas.

Enzymatic source of H_2. Several experiments were performed to characterize the enzymatic nature of the H_2-evolving hydrogenase. As shown in Fig. 9, the rate of H_2-production was linearly related to the cellular activity of nitrogenase. This was true for each of the three carbon sources employed (malate, lactate and glucose).

In a second series of experiments, nitrogen and acetylene were compared for their effects on H_2 production by nitrogen limited cells. As can be seen (Fig. 10), H_2-production from glucose was maximal under the inert atmosphere of Ar (upper curve). The middle curves were obtained using gas phases of argon + 5% (v/v) acetylene, or purified N_2. The surface areas for the argon-acetylene and N_2 mixtures were 17 and 2 cm^2, respectively. Both gas phases inhibited H_2 production to a level about 50% of the values obtained for a pure argon atmosphere.

To some extent, the inhibitions caused by N_2 plus acetylene appeared to be cummulative, as shown by the curve for N_2-gas plus 5% acetylene (surface area of 2 cm^2) and about 75% relative to the argon culture. However,

the strongest inhibition of H_2 synthesis occurred in the culture incubated under N_2, but with the surface area increased to 17 cm^2. Thus, in situ H_2 production by R. sphaeroides was inhibited by both acetylene and N_2. Carbon monoxide was also added to cultures actively producing H_2 gas (Fig. 11). As can be seen, acetylene reduction was completely suppressed by CO, but the rate of H_2 production was only slightly, if at all, reduced by this gas. Similar cultures exposed to 18% O_2 in argon as the atmosphere showed the irreversible loss of both H_2-evolving and acetylene-reducing capacities (Fig. 12).

Stoichiometry. Cultures of the glucose-utilizing strain (glc+) and of the wild-type (w.t.) were assayed quantitatively (system (ii)) for substrate conversion using radioactively-labeled compounds (Table I). The wild-type cells converted 24% of the available glucose to the equivalent amounts of H_2 (see eqn. 1). $^{14}CO_2$ 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 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 (see eqn. 2). Lactate as the carbon source for the wild-type gave 48% conversion to H_2 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 go H_2, with 91% conversion to CO_2, and with no apparent substrate remainign after H_2 production had ceased. It should be noted that the extent of glucose-supported H_2 production by glc+ was much greater than for the wild-type R. sphaeroides.

Table 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 (Table 2). 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 (eqn. 1). The w.t. strain converted 9% of the available glucose to the equivalent amount of $H_2 + CO_2$. In both cases, as atmosphere of molecular nitrogen 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 H+ions. NH_4Cl at a concentration of 5 mM completely suppressed H_2 production in the modified Hungate tubes, along with inhibition of CO_2 production.

The possible identity of low molecular weight compounds formed during H_2 production (Table I) was investigated by GLC analysis (Fig. 13). Spent culture suspending media of the mutant glc+ and the wild-type were trimethylsilylated and analyzed by glc in these experiments. The wild-type organism (w.t.) formed no discernible soluble product and only the two isomeric forms of glucose were found at the completion of H_2 production. Mutant glc+ converted some of the glucose to gluconate and an unknown compound migrating with a retention time nearly identical to the 5-carbon sugars and we have termed this unknown material as "C-5-sugar".

DISCUSSION

Nitrogenase. The data in this paper supports the concept that H_2 biosynthesis in Rps. 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 nitrogenase, inhibited H_2 evolution. As might be expected, increasing the surface area of the cultures exposed to these compounds increased the inhibitory effect. Second, ammonium ion, which is generally a repressor of nitrogenase synthesis (15), blocked the synthesis of H_2. Third, the cellular specific actifity of nitrogenase was coordinately (linerly) related to the specific activity of the H_2 producing enzyme. And, finally, the effects of CO on in-situ H_2 biosynthesis were predictable, based on published results with the purified enzyme (9). As reported by others, CO blocks nitrogenase catalysed reduction of molecular nitrogen without inhibiting H_2 evolution (8,9). We have demonstrated a similar effect for Rps. sphaeroides.

Mutants. It was determined that glucose did not support extensive H_2 production by the wild-type. However, selection of the glc+ and gln+ mutants capable of H_2 production was accomplished without difficulty by successive subculturing on a mineral base medium containing glucose or gluconate as the sole carbon source. These mutant strains are probably altered in their increased capacity to convert glucose to C3 metabolites using the steps of the Entner-Doudoroff pathway, the principle degradative pathway for glucose (3,17). It is of interest, that the glc+ mutant excreted significant quantities of a metabolite migrating with the same solubility (paper chromatography) and retention time (GLC) as gluconate, an intermediate in the Entner-Doudoroff pathway. Evidently, the oxidation of glucose to gluconate preceeds 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.

FIGURE LEGENDS

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

Fig. 2. 100 ml culture flask with attached CO_2 trap and manometer. Trap is filled with 0.1N NaOH.

Fig. 3. H_2 evolution by wild-type Rps. sphaeroides. Wild-type cultures were grown in minimal medium with 10mM of the indicated carbon source anaerobically at 800 ft candles illumination. O—O glucose, Δ—Δ lactate, ●—● malate, □—□ gluconate, ■———■ citrate. (A) rate of H_2 evolution vs. time. (B) total H_2 evolved vs. time.

Fig. 4. H_2 evolution by glc+ and gnt+ mutants of Rps. sphaerodies. The appropriate strains were grown anaerobically in minimal medium with 10mM of the indicated carbon source at 800 ft candles illumination. 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.

Fig. 5. Maintenance of H_2evolution rate of R. sphaeroides. Wild-type R. sphaeroides was grown anaerobically on minimal medium with 20mM latate as carbon source in the light. Lactate equivalent to 20mM concentration was added as the H_2-evolution rate fell (arrows).

Fig. 6. Maintenance of H_2 evolution rate of R. sphaeroides. Wild-type R. sphaeroides was grown anaerobically on minimal medium with 10mM malate as carbon source in the light. Malate equivalent to 10mM concentration was added every other day. Replenishment of nutrients was at arrows. O—O no replenishment. □——□ yeast extract (0.02%) added, Δ——Δ conc. Hutner's base (2.0%) added, ●—● yeast extract (0.02%) and conc. Hutner's base (2.0%) added.

Fig. 7. Effect of ammonium ion on H_2 production by wild-type Rps. sphaeroides. Wild-type Rps. sphaeroides was grown anaerobically in the light on minimal levels of NH_4Cl. (A) Ammonium ion levels vs. time, (B) optical density vs. time, (C) H_2 evolution rates vs. time. ▲——▲ 0.5mM NH_4^+, ■——■ 2.0mM NH_4^+. Δ——Δ 6.0mM NH_4^+, □——□ 10mM, NH_4^+, ●—● 20mM NH_4^+.

Fig. 8. Light intensity and H_2 evolution. Wild-type cells were grown anaerobically in minimal medium with 20mM lactate as carbon source.

Fig. 9. Comparison of H_2 evolution with acetylene reduction. Wild-type and Glc+ mutants were grown anaerobically on minimal medium with 20mM of the indicated carbon source in the light. Δ malate, wild-type; ▲ lactate, wild-type, ● glucose, glc+.

Fig. 10. Hydrogen production vs. gas phase. Glc+ cells were grown in system (iii) tubes on glucose anaerobically in the light. Atmospheres were maintained as indicated by daily flushing and injection of the appropriate purified gases. Surface area was increased by tilting the tubes on their sides. ●—● Ar atmosphere; ■—■ Ar + 50% acetylene atmosphere; ▼—▼ N_2 atmosphere (2 cm^2 surface

area); O—O N_2 + 50% acetylene atmosphere (2 cm^2 surface area); ▲— N_2 atmosphere (17 cm^2 surface area).

Fig. 11. Effect of carbon monoxide. Cells were grown under Ar in minimal medium with 10mM malate in the lgiht. After the onset of gas evolution, the atmosphere was made 10% in CO (dark bar). Controls under Ar:△—△ H_2 evolution, O-O C_2H_2 reduction. Cultures with 10% CO:▲—▲ H_2 evolution, ●—● C_2H_2 reduction.

Fig. 12. Effect of oxygen. Cells were grown under Ar in minimal medium with 10mM malate in the light. After the onset of gas evolution, the atmosphere was made 18% in O_2 (dark bar). After the period under O_2, the atmosphere was flushed with Ar. Controls under Ar: △ - △ H_2 evolution, O - O C_2H_2 reduction. Cultures with 18% O_2:▲—▲ H_2 evolution, ● - ● C_2H_2 reduction.

Fig. 13. GLC Analysis of trimethylsilyl derivatives of culture media. After gassing, cultures were freeze dried, trimethylsilylated and separated by GLC using a flame ionization detector (FID). (A) glc+ strain, (B) w.t. strain.

Table I

cell type	w.t.	w.t.	w.t.	glc+
substrate	glucose (0.5 mmole)	malate (0.5 mmol)	lactate (1.0 mmole)	glucose (1.0 mmol)
H_2 gas evolved	1. 4 mmol (24%) ave.	1.7 mmol (57%) ave	2.9 mmol (48%) ave.	12.0 mmol (99%) ave
total CO_2 recovered	1.0 mmol (34%) ave	1.25 mmol (60%) ave	1.7 mmol (60%) ave.	5.45 mmol (91%) ave
total label recovered	91% ave	92% ave	91% ave	95% ave

All cultures grown in minimal medium with 0.2% yeast extract as nitrogen source and 10 mM for carbon source, pH 7.2. Other conditions as described.

Gas phase CO_2 5-17%.

Table 2

H_2 Yields from Glucose: Strains glc+ and w.t.

Sample	Surface Area	Nitrogen Source	H_2[1]	CO_2[1]	Ratio	% Conversion[2]
glc+-Ar[3]	17 cm^2	0.05% Yeast Extract	435.6	197.3	2.21	36
glc+-N_2[4]	2 cm^2	" "	167.1	128.5	1.30	14
w.t.-Ar[5]	17 cm^2	" "	98.7	98.2	1.02	9
w.t.-N_2[5,6]	2 cm^2	" "	32.9	32.4	1.02	3
glc+-N_2	17 cm^2	" "	0	117.3	0	0
w.t.-Ar	17 cm^2	5mMHN_4Cl	0	34.2	0	0

[1]Values for CO_2 and H_2 are the average of three replicate samples and are expressed in terms of µmoles per mg dry weight of cells.

[2]Percent conversion based on the possible complete photo dissmimlation of 100 µmoles of glucose: 10 $C_2H_{12}O_6$+600 H_2O = 600 CO_2 + 1200 H_2.

[3]Mutant glc+, Ar atmosphere

[4]Mutant glc+ N_2 atmosphere

[5]w.t. Ar atmosphere

[6]w.t. N_2 atmosphere

Table 3

Carbon Recovery After Photo-Dissimilation of Glucose

Sample	CO_2[1]	Cells	Supernate	Percent	Remaining Substrate
glc+-Ar[1]	32.9	15.5	44.4	92.8	25.0
glc+-N_2	19.6	31.5	31.8	82.9	0
glc+-Ar plus NH_4Cl	5.7	20.8	54.0	80.5	6
w.t.-N_2	5.4	18.5	69.9	93.8	0
w.t.-N_2 plus NH_4Cl	5.8	23.7	56.0	85.5	26.0

[1]The values for carbon are the average of three replicate samples, and are expressed in terms of the initial amount of glucose (100 µmoles) present in each sample. Each sample contained 5 ml of HMB medium (methods) supplemented with 20 µmoles/ml glucose and 0.05% w/v yeast extract as a limiting amount of combined nitrogen. The specific activity of the substrate was 1100 dpm/µmole ^{14}C. The carbon in columns 1-4 was determined on the basis of ^{14}C-measurements; remaining substrate (column 6) by gas liquid chromatography (methods). Symbols and conditions are the same as in Table 2.

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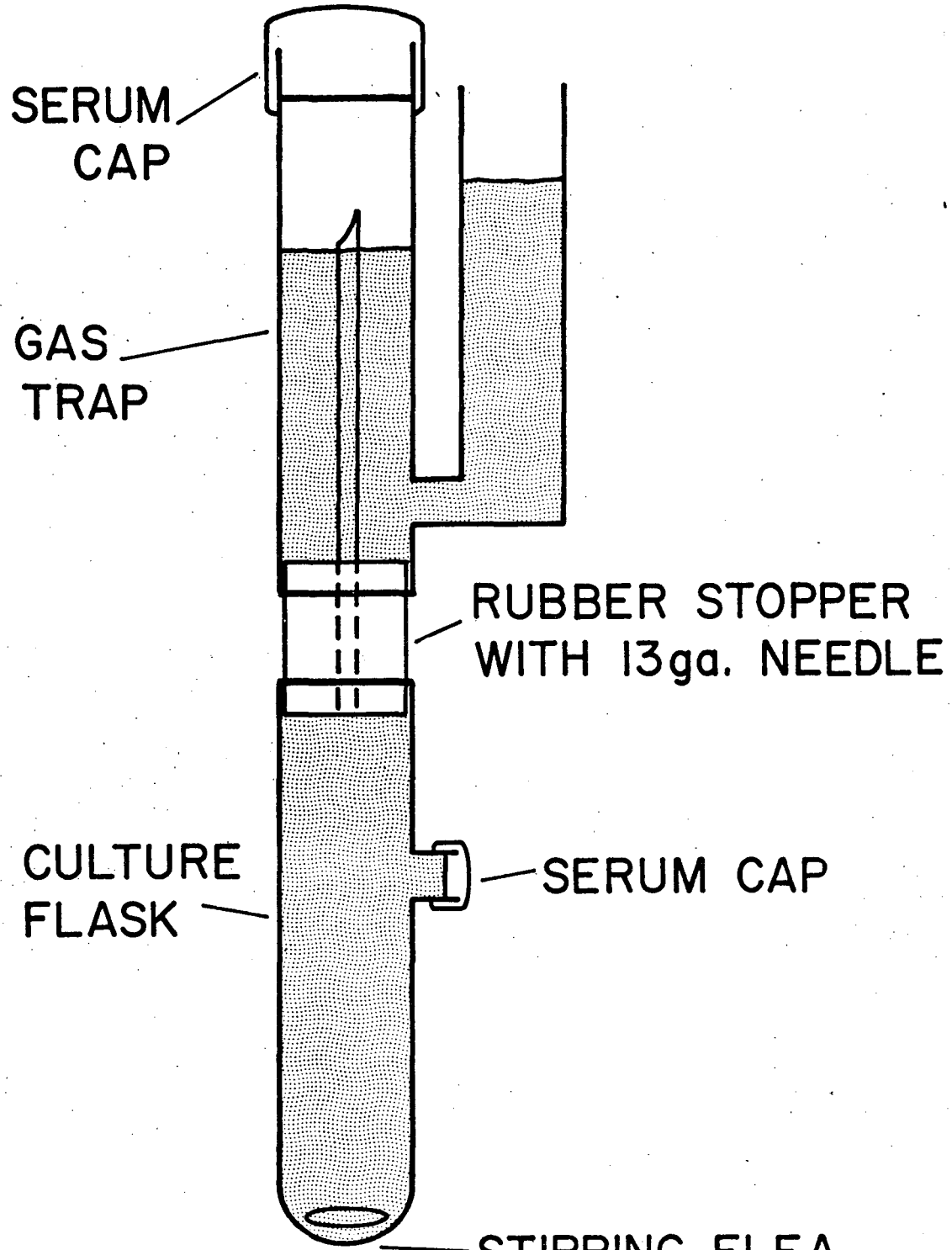
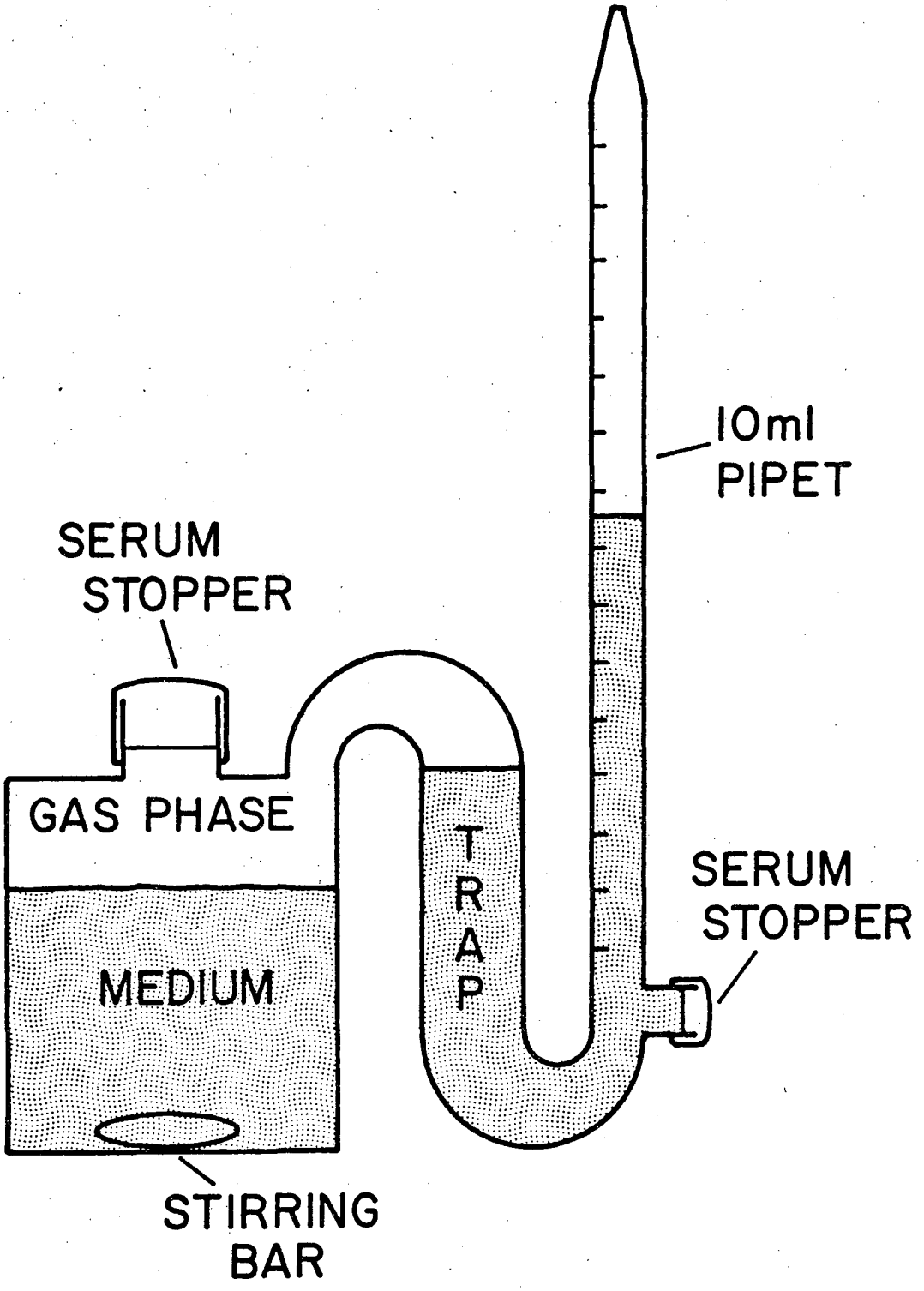
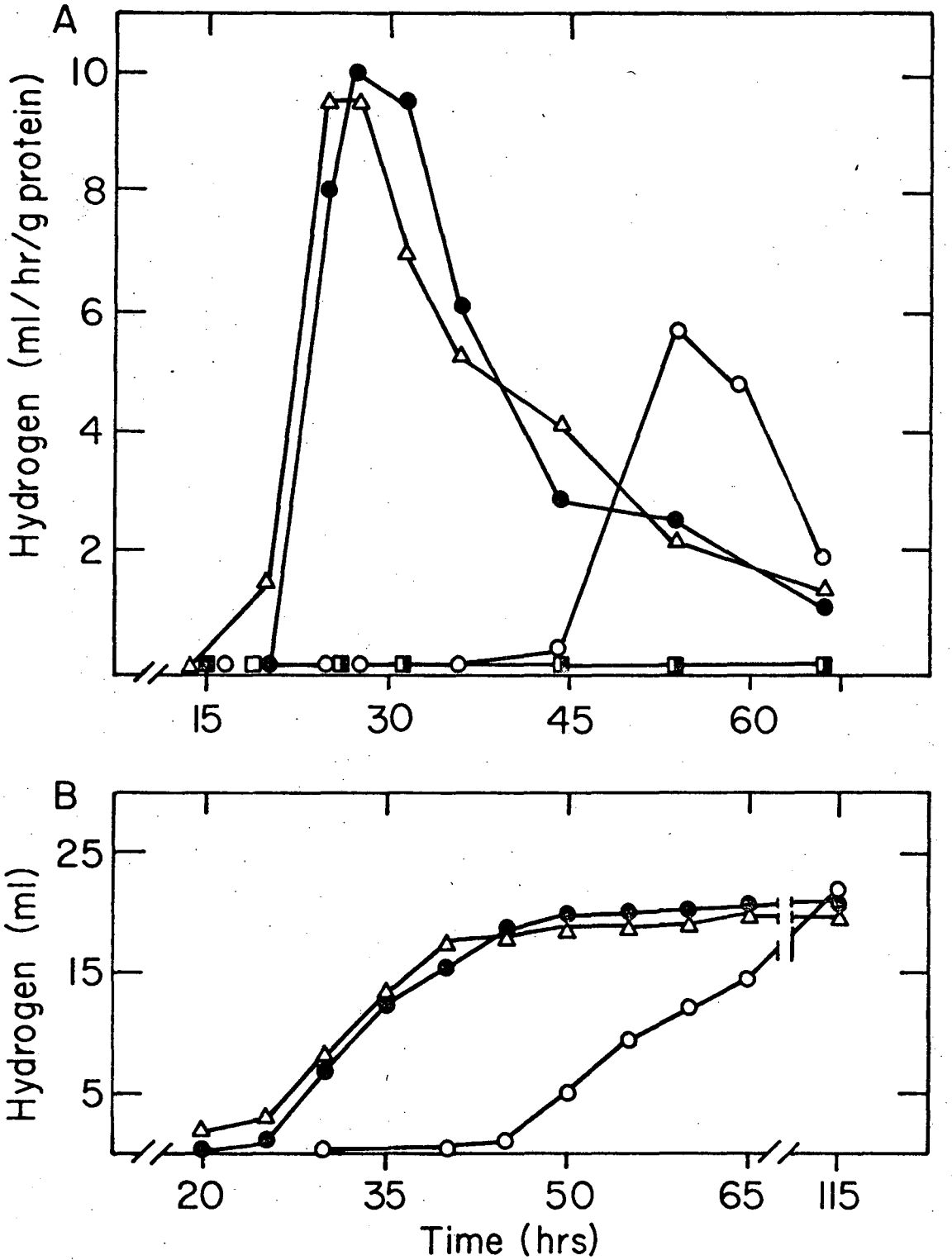

SERUM CAP
GAS TRAP
RUBBER STOPPER WITH 13ga. NEEDLE
CULTURE FLASK
SERUM CAP
STIRRING FLEA

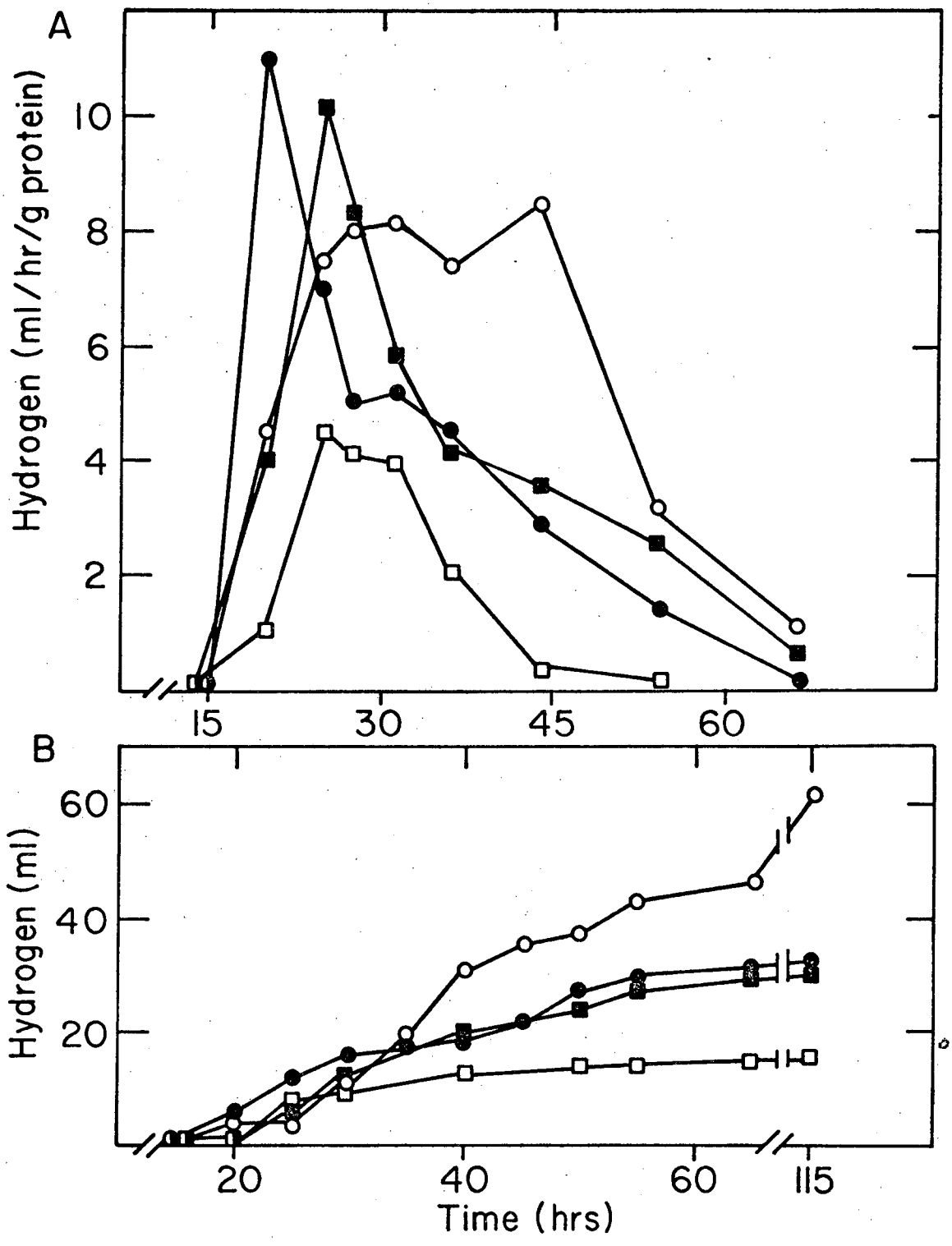
XBL 782—3813


10ml
PIPET
SERUM
STOPPER
GAS PHASE
MEDIUM
TRAP
SERUM
STOPPER
STIRRING
BAR

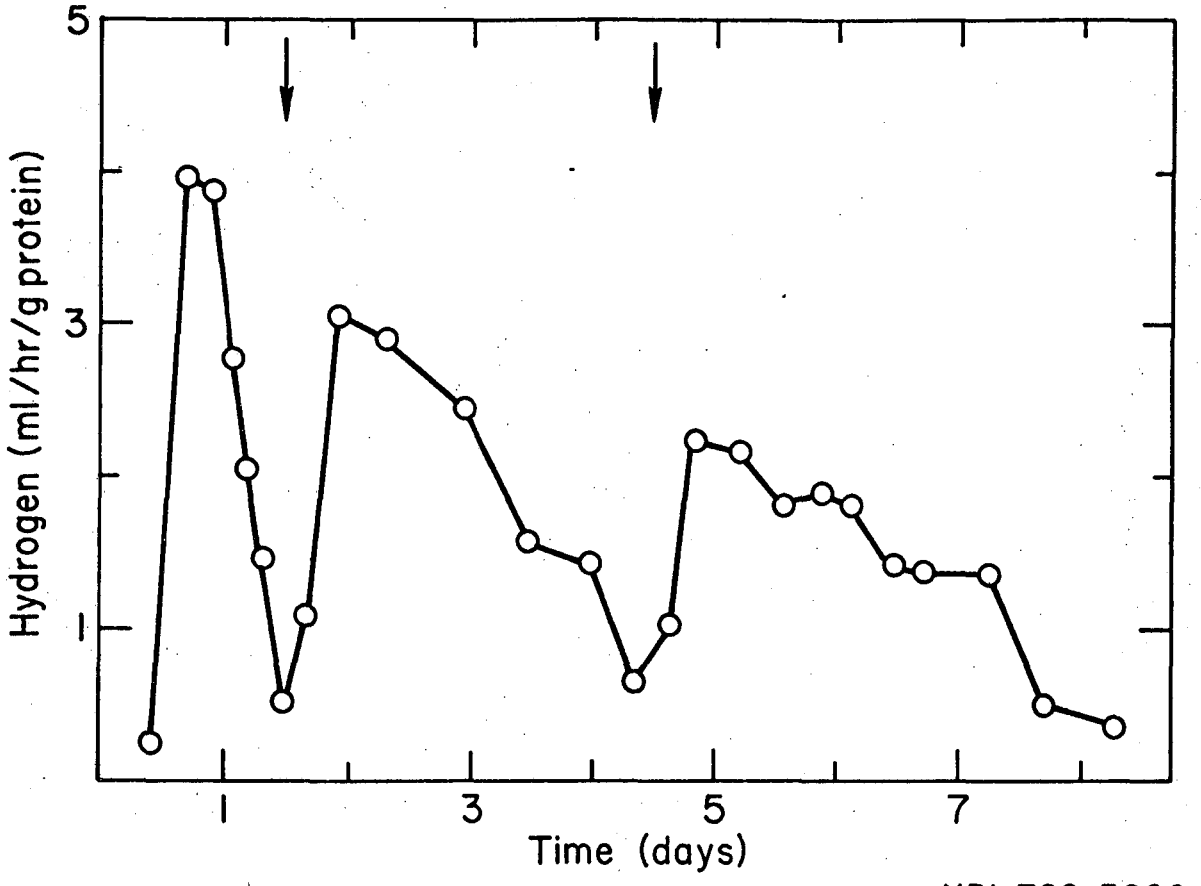
XBL782-3812


A
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


A
Hydrogen (ml/hr/g protein)
10
8
6
4
2
15
30
45
60
B
Hydrogen (ml)
60
40
20
20
40
60
115
Time (hrs)

XBL789-4221



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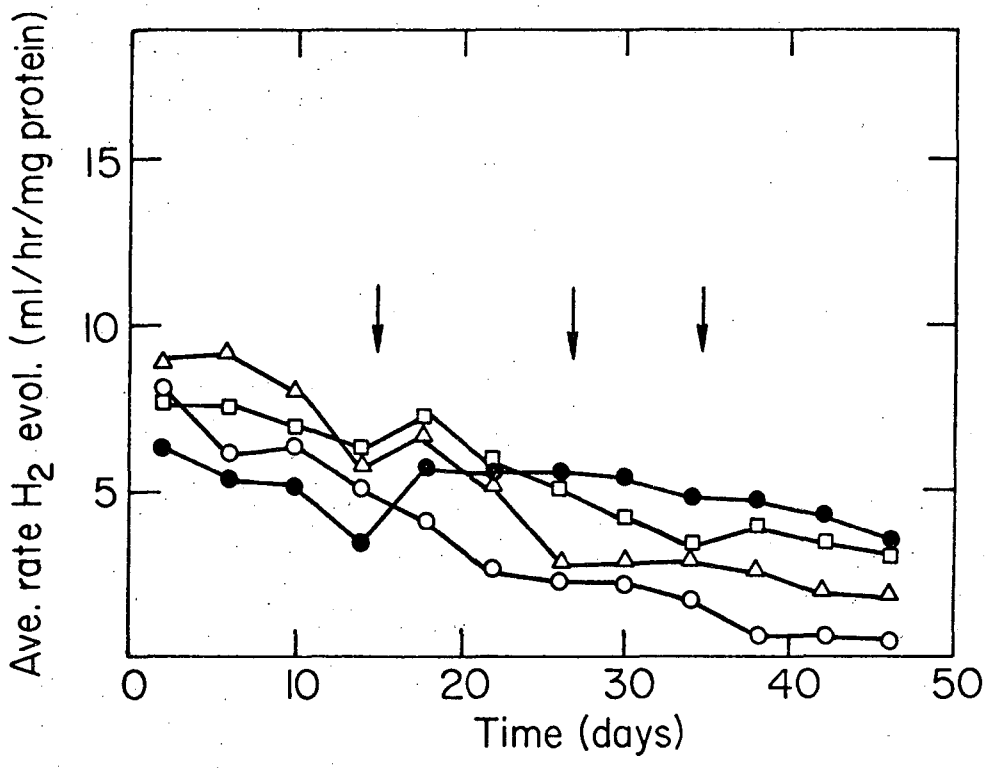
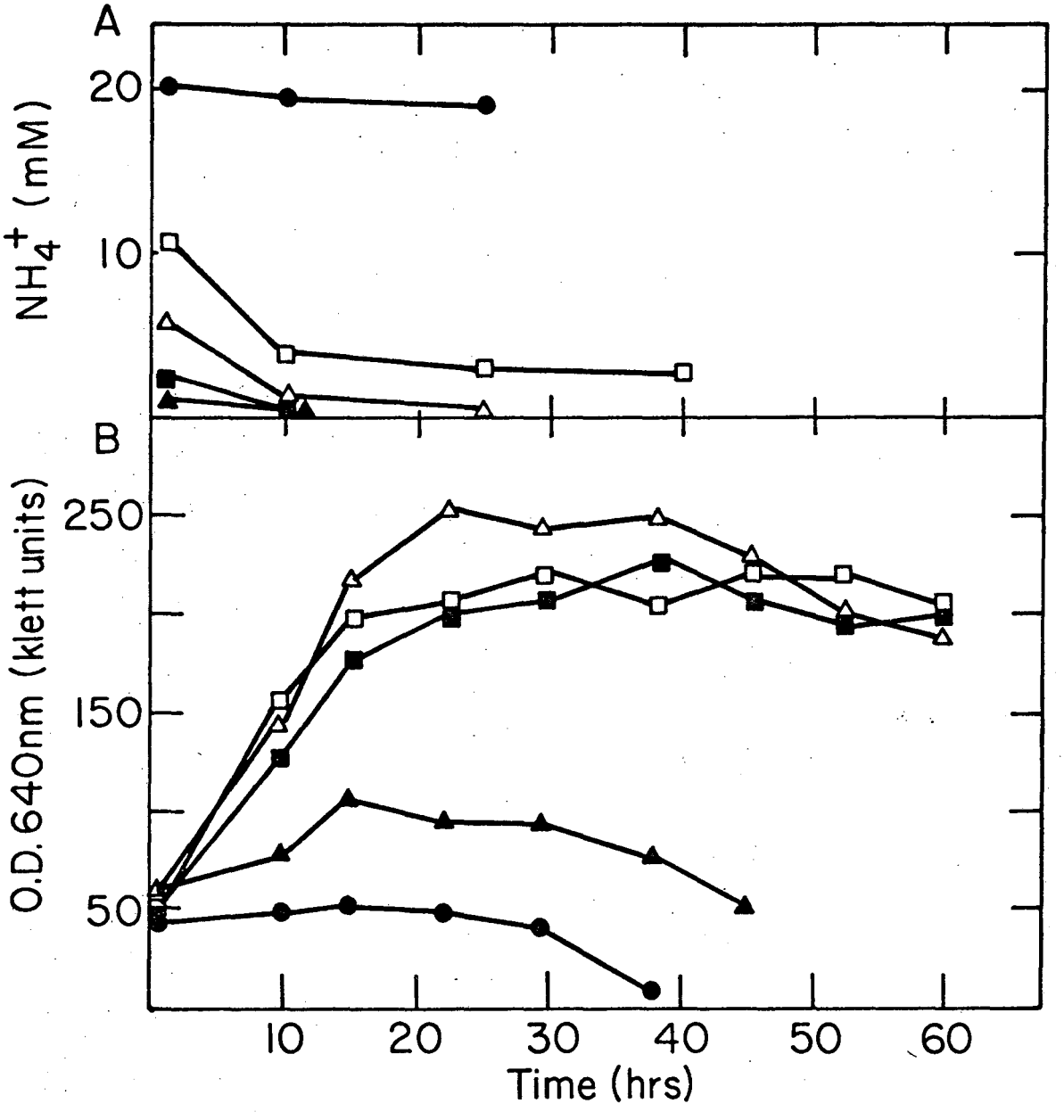
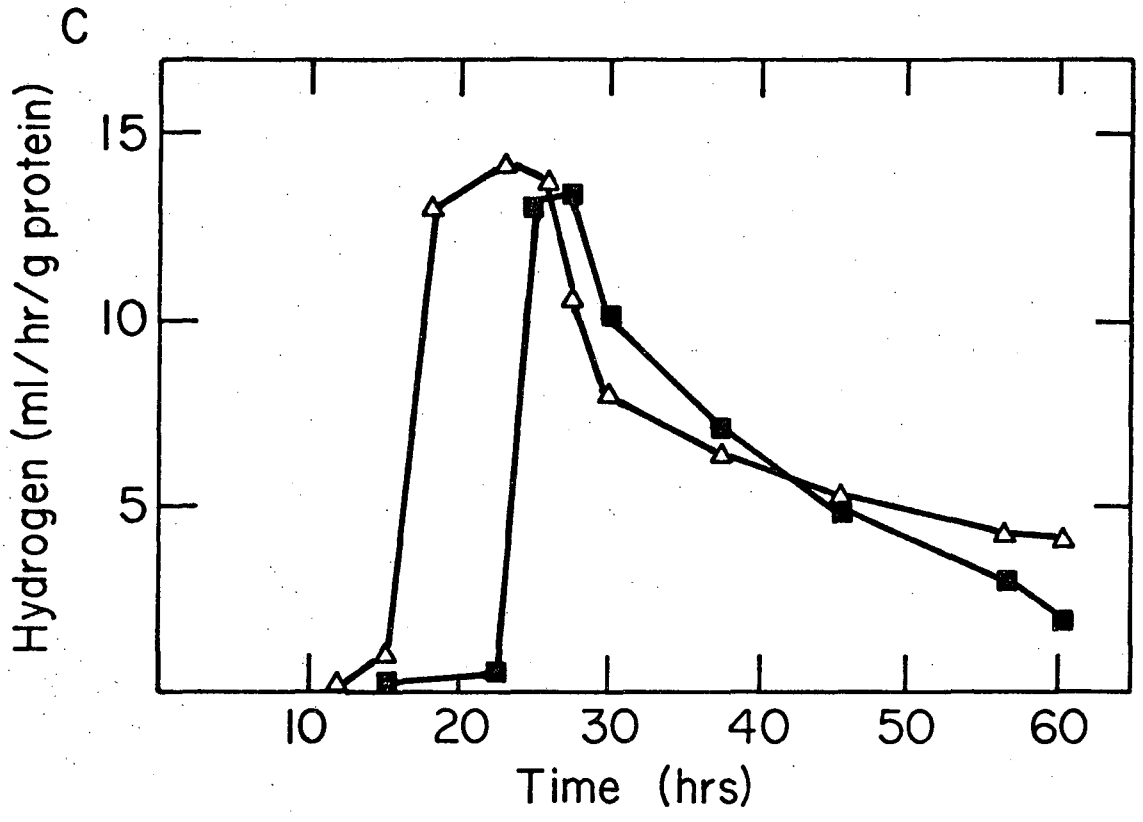

Ave. rate H_2 evol. (ml/hr/mg protein)
15
10
5
0
10
20
30
40
50
Time (days)

XBL 789-4215


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

XBL 789-4219



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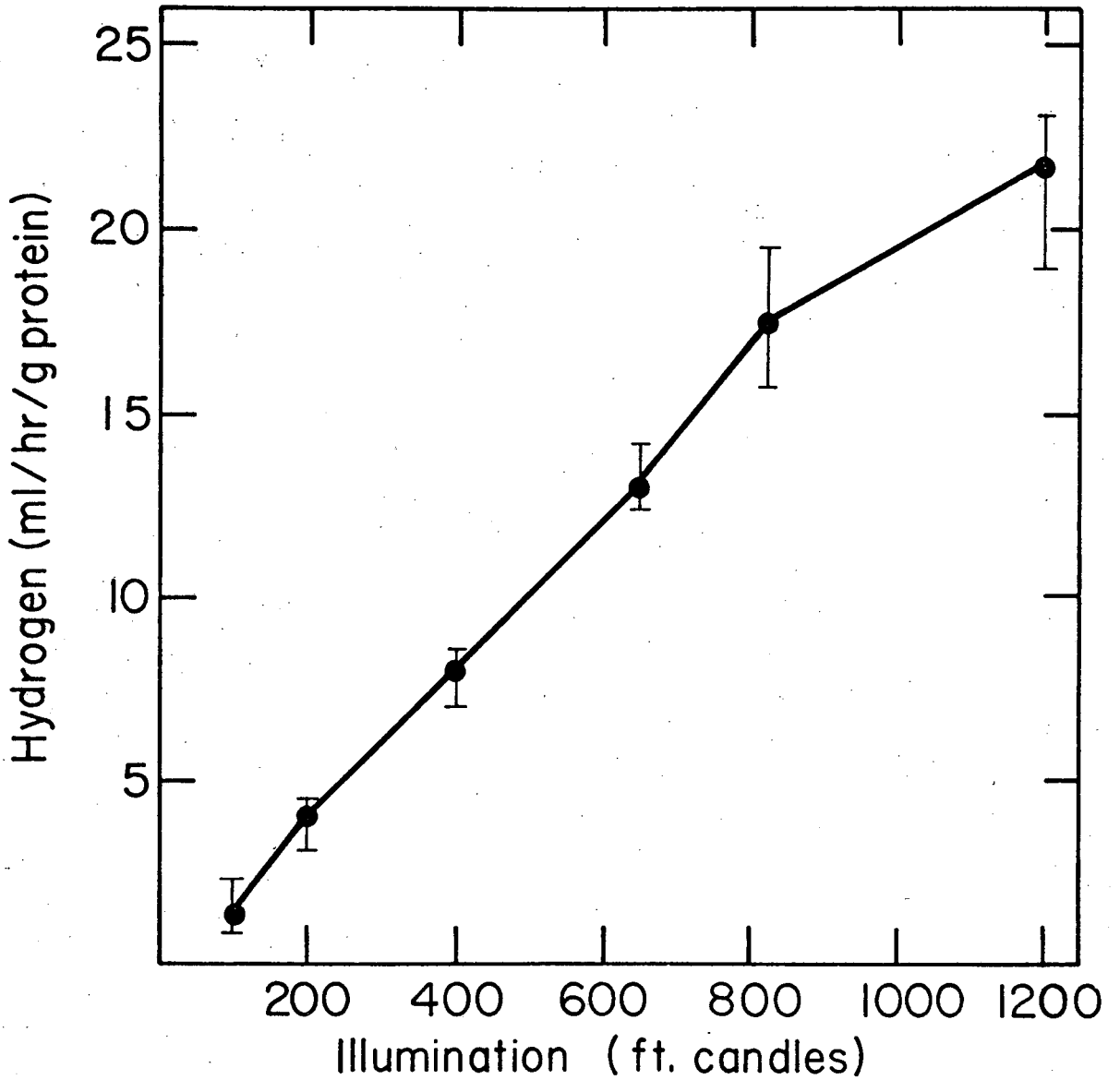
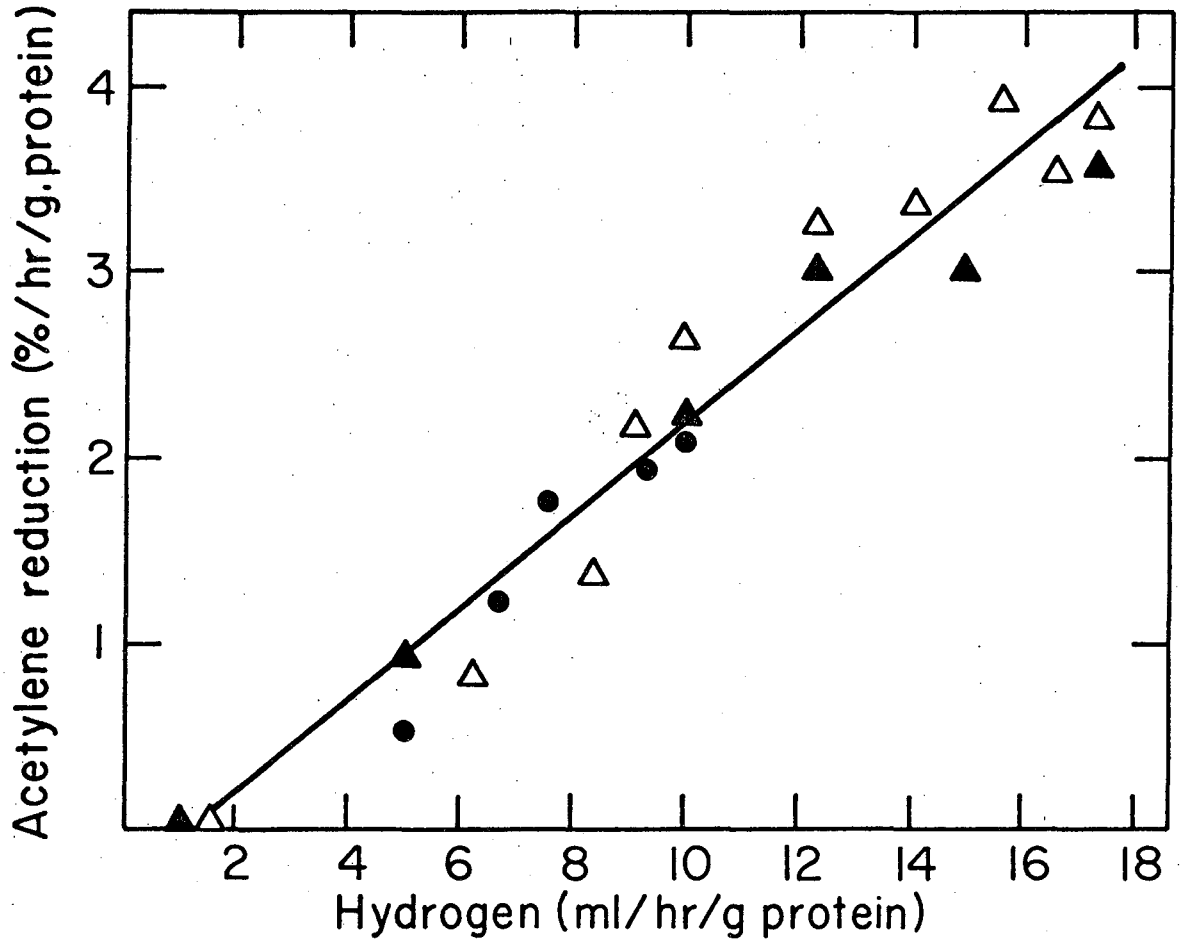
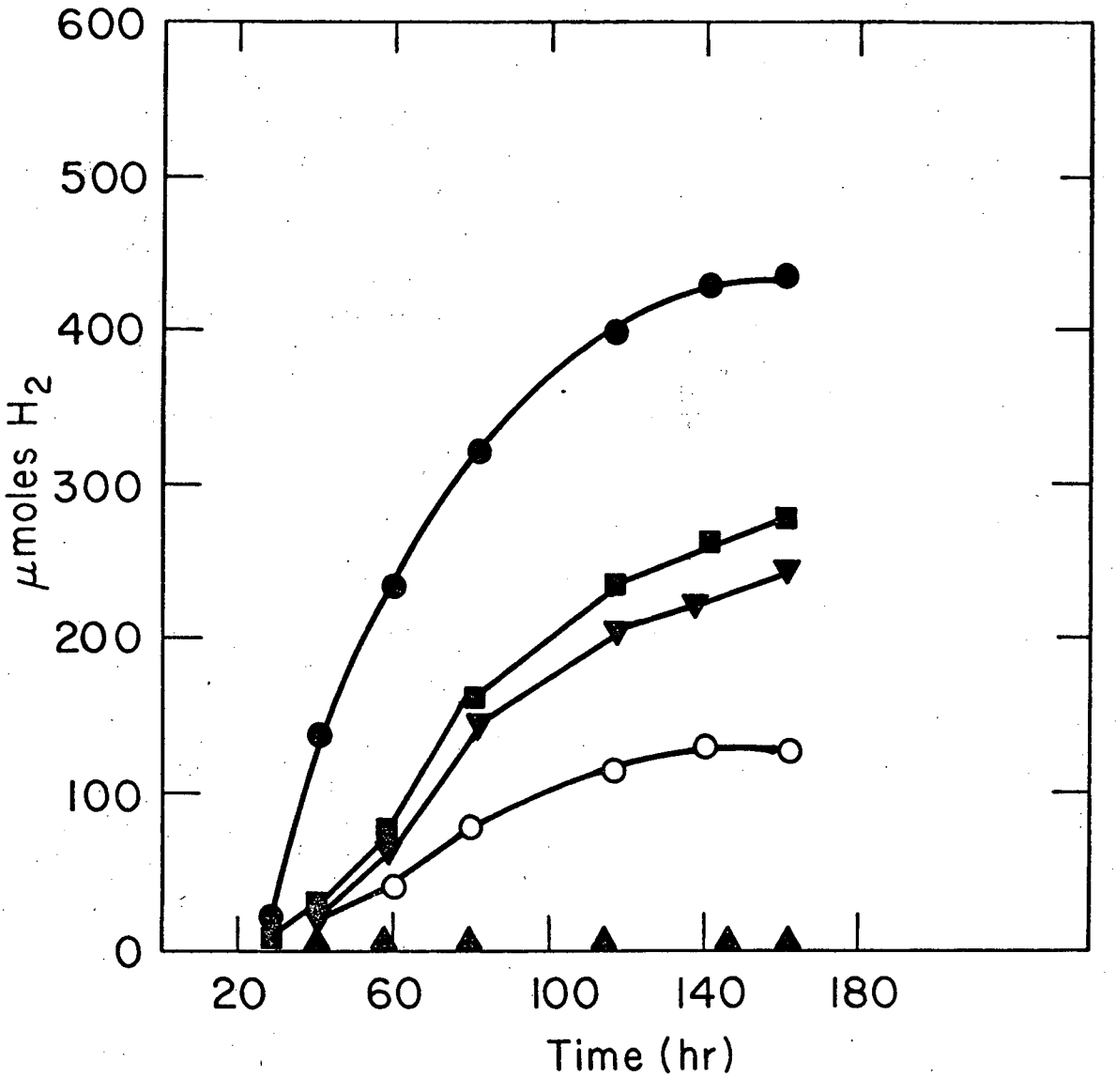

25
20
15
10
5
Hydrogen (ml/hr/g protein)
200
400
600
800
1000
1200
Illumination (ft. candles)

XBL 782-3793


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

XBL789-4217



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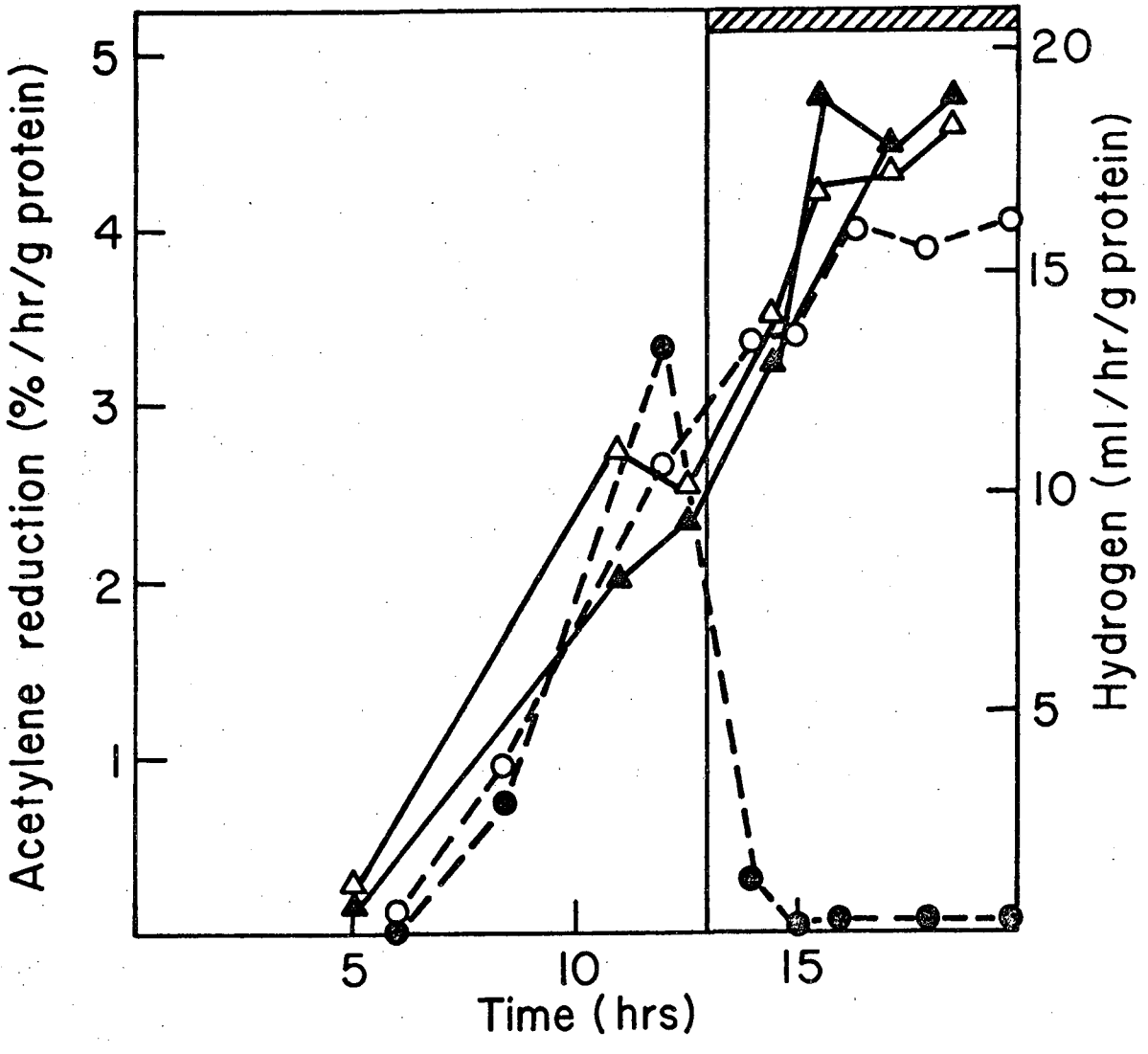
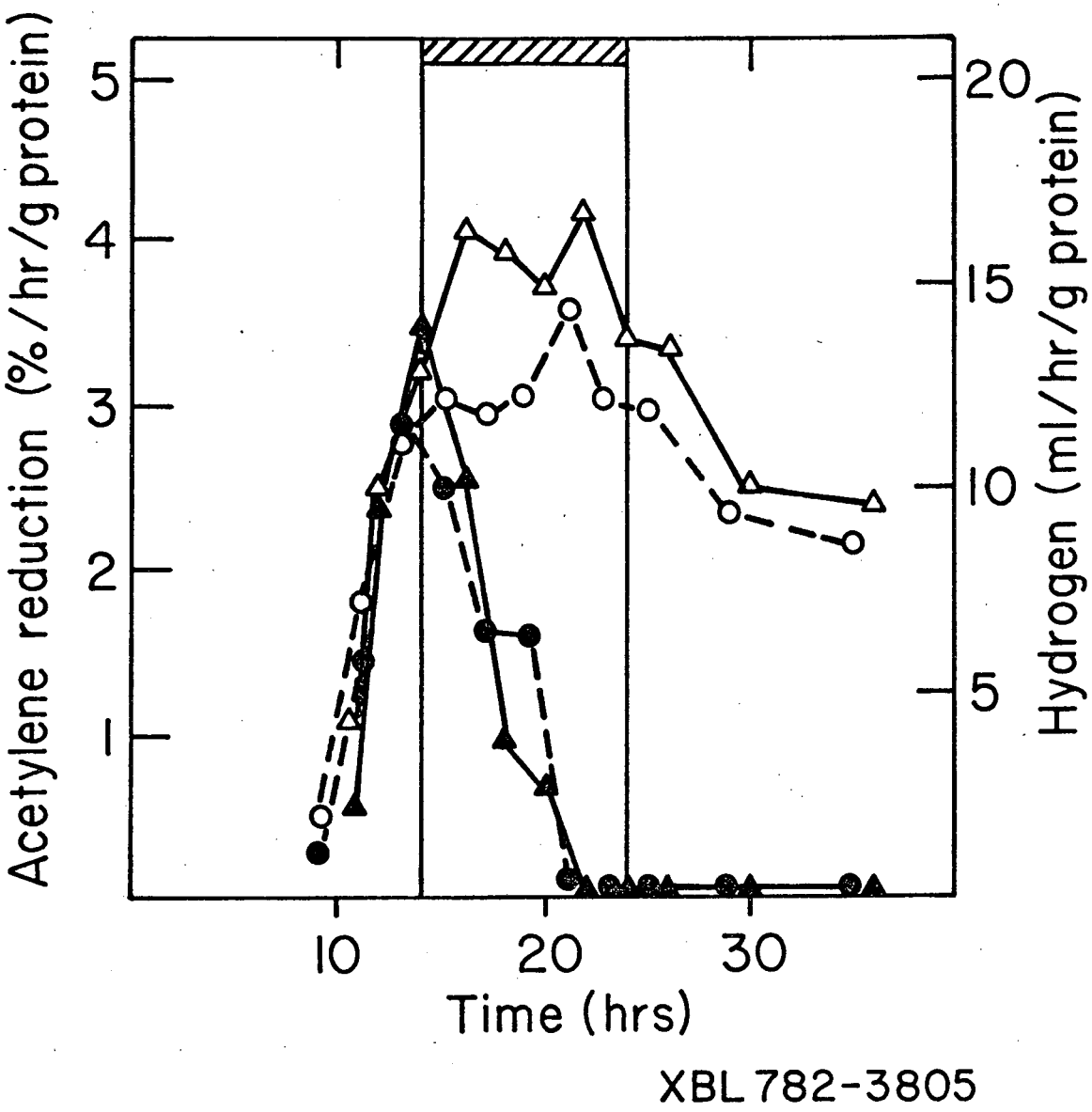
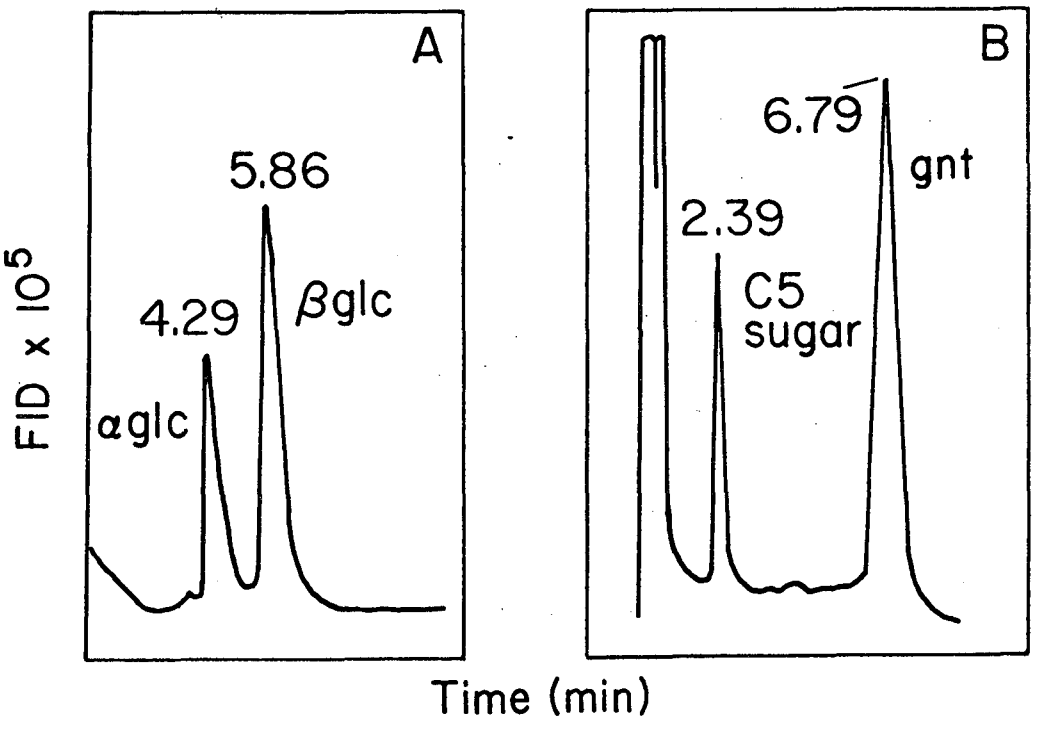

Acetylene reduction (% /hr/g protein)
Hydrogen (ml/hr/g protein)
Time (hrs)
1
2
3
4
5
5
10
15
20
5
10
15

XBL789-4216


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

XBL 782-3805



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This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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