JOURNAL OF BACTERIOLOGY, May 1979, p. 446-452
0021-9193/79/05-0446/07$02.00/0

Vol. 138, No. 2

Hydrogen Formation in Nearly Stoichiometric Amounts from Glucose by a *Rhodopseudomonas sphaeroides* Mutant

BRUCE A. MACLER,[1]* RICHARD A. PELROY,[2] AND JAMES A. BASSHAM[1]

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720,[1] and Battelle Pacific Northwest Laboratories, Richland, Washington 99352[2]

Received for publication 19 October 1978

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 adenosine 5'-triphosphate. 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, nonsulfur photosynthetic bacteria produce molecular hydrogen under defined conditions (5). Although low rates of hydrogen biosynthesis occur 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 under certain conditions 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:

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

$$C_4H_6O_5 + 3\ H_2O \xrightleftharpoons{\text{light}} 6\ H_2 + 4\ CO_2 \quad (2)$$

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, photoelectron transport, or both. Although the standard degradative pathways (oxidative pentose phosphate cycle, tricarboxylic acid cycle, and Entner-Doudoroff pathway) occur in these organisms (1, 3), the specific pathways responsible for hydrogen production have not been identified.

Hydrogen formation can be mediated by nitrogenase or 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. Hydrogenases are noncompetitively inhibited by carbon monoxide, which may affect the obligatory electron transport chain. Cytochrome c_3 is generally 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 to serve as hydrogenase substrates.

Nitrogenases are irreversibly inhibited by oxygen. They are noncompetitively 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 nonsulfur 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 *Rhodopseudomonas capsulata*. We present here similar results for *R. 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 *R. 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 *R. sphaeroides* 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% (vol/vol) Hutner's concentrated mineral base with growth factors (2) and 1% (wt/vol) yeast extract (Difco Laboratories)–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% (wt/vol) 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 General Electric Lumiline incandescent bulbs in a temperature-controlled light box. Temperature was maintained at 27 to 28°C. 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). Ammonia was determined colorimetrically, using a Nessler's reagent assay (Sigma Chemical Co.).

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 0.01 N 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 of minimal medium and a gas phase of known volume, 50 to 70 ml. The trap contained 0.1 N 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. (iii) In the third system, cells for H_2 production were grown in Hungate-type tubes (Bellco Glass, Inc.) equipped with flange-type butyl-rubber stoppers to allow sampling of the gas phase above the cultures or the contents of the liquid medium. 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 to 5% by volume of a stationary-phase culture, and the flasks were assembled and flushed with the appropriate atmosphere.

For all systems, the constituents of the collected gases were determined qualitatively and quantitatively by gas-liquid chromatography (GLC), using a modified Aerograph 1520 gas-liquid chromatograph with columns of Porapak R, Porapak T, or a 0.5-nm 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 gases were helium and nitrogen, respectively, for the two systems. It was found that the alkaline traps of system i and ii removed

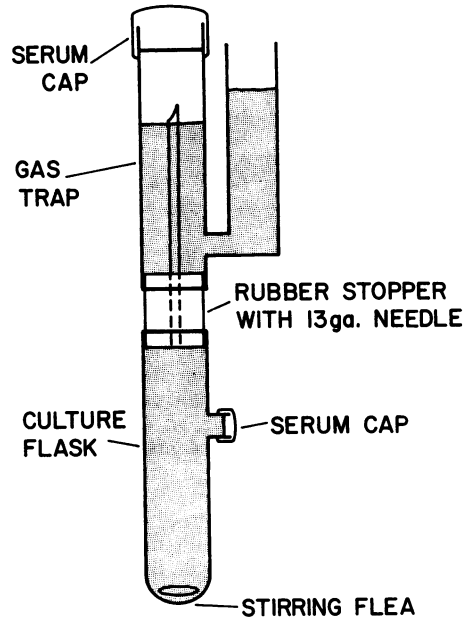

FIG. 1. *Culture tube (25 ml) with attached CO_2 trap. Trap is filled with 0.01 N NaOH.*

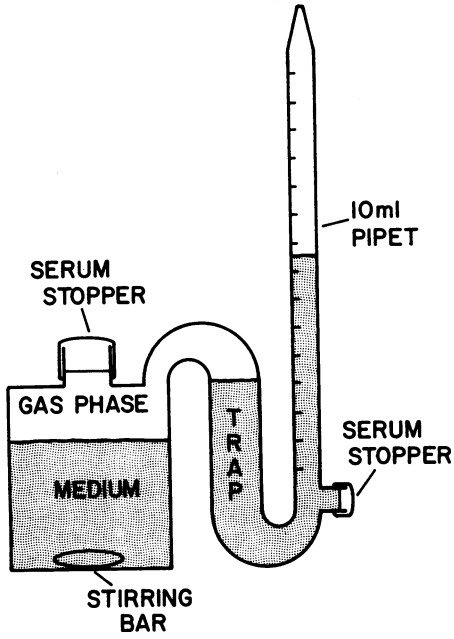

FIG. 2. *Culture flask (100 ml) with attached CO_2 trap and manometer. Trap is filled with 0.1 N NaOH.*

85 to 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 5 min to standardize atmospheres. This was sufficient as shown by 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 gastight syringes and assayed by GLC for ethylene as described above, using a column of Porapak T at 175°C 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 by 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 over 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 × *g* for 5 min, then suspended in 25 ml of minimal medium in the 25-ml tubes with attached traps (system i). The culture medium was made up to 20 mM in the appropriate carbon source, and the flasks were allowed to incubate at 27°C and 10,000-lux illumination. As gas production began (10 to 20 h), low levels (0.4 to 0.8 mCi/mmol) of the appropriate uniformly ^{14}C-labeled carbon source (New England Nuclear Corp.) were added to the cultures. The gas was measured and collected at intervals and injected into serum-stoppered Warburg flasks containing 2 N KOH in the center wells to trap atmospheric CO_2. After gas evolution ceased, the culture media were collected and placed inside wells of Warburg flasks containing 2 N 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. Portions were assayed by two-dimensional paper chromatography, autoradiography, and Geiger counting (10, 16). The solvent system for the first dimension was phenol–water–glacial acetic acid–EDTA (840:160:10:1, vol/vol/vol/vol) and in the second dimension equal volumes of *n*-butanol–water (370:25, vol/vol) and propionic acid–water (180:220, vol/vol). Chromatograms were developed for 36 h in each direction.

CO_2 production using system iii was assayed in a similar manner by quantitative liquid scintillation counting of $^{14}CO_2$ recovered from supernatant fractions of spent media after hydrogen production. Uniformly labeled malate and glucose were used as substrates. 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 supernatant fractions were then added to a Warburg vessel containing 0.4 ml of an organic base (NCS tissue solubilizer; Amersham/Searle) in the center well. A 1-ml amount of 4 N HCl was then added to the main chamber of the vessel, and the vessel was 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 was 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 by 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 four times with the growth medium, and portions 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 GLC 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 Co.) or with TMS samples prepared by the method described by Laine et al. (12). The FID response was linear from 0.01 to 0.1 μg 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 to 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, and silylating reagents were added to the dried residue containing the nonvolatile by-products of the spent medium. The reaction mixture was then stored at room temperature for about 6 h 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 to 2.0 μl of the silylation mixture directly onto the column of the gas chromatograph.

RESULTS

Isolation of permissive strains. To isolate strains of *R. sphaeroides* able to produce H_2 gas from glucose or gluconate from wild-type strains which do not ordinarily produce H_2 gas, the wild-type cells were spread on 2% (wt/vol) agar

plates made with rich medium and 10 mM glucose or gluconate. These plates were grown aerobically in the light. The small, pigmented colonies that appeared 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 inoculate minimal media and 10 mM carbon source in 25-ml tubes with traps. These tubes were plated in the light at 27°C. 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 *R. sphaeroides* utilizes malate and lactate efficiently, both in terms of maximum rate and in terms of total conversion (Fig. 3). With glucose as substrate, however, wild-type organisms form little H_2, and only after a considerable lag. Gluconate yields no H_2. Both compounds support dense photosynthetic growth, however. Citrate supports no growth on minimal medium alone, and such cultures do not produce H_2. The Glc^+ strain produces 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 70 h (Fig. 3a and 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. 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 6 weeks, although the rate of gas evolution was reduced.

Culture conditions versus H_2 production. Ammonium concentration and light intensity were both important in determining rates of H_2 production by *R. sphaeroides*. Either comparatively high (20 mM) or low (0.5 mM) levels of NH_4Cl did not support or only poorly supported

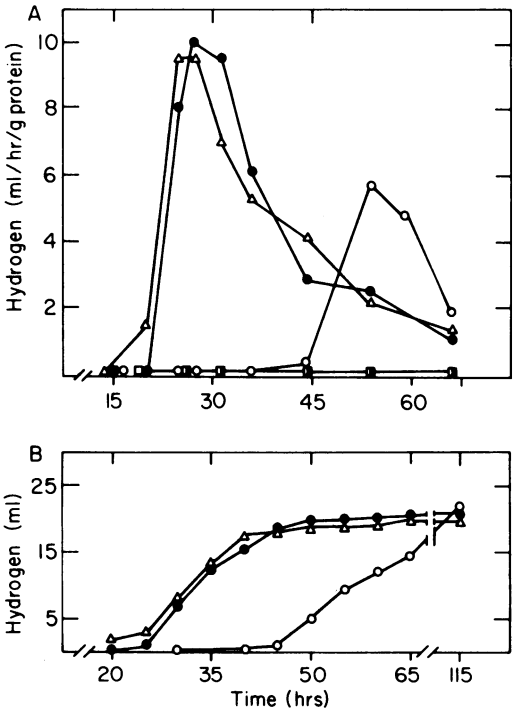

FIG. 3. *H_2 evolution by wild-type R. sphaeroides. Wild-type cultures were grown in minimal medium with a 10 mM concentration of the indicated carbon source anaerobically at 8,000-lux illumination. Symbols: ○, glucose; ▲, lactate; ●, malate; □, gluconate; ■, citrate. (A) Rate of H_2 evolution versus time; (B) total H_2 evolved versus time.*

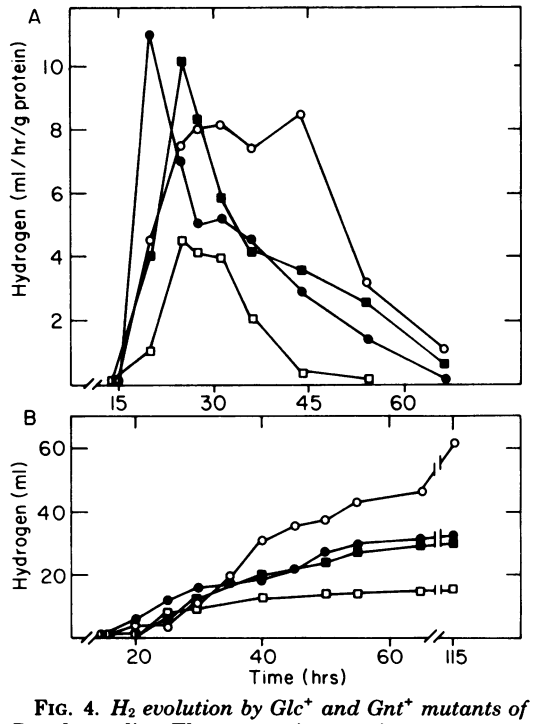

FIG. 4. *H_2 evolution by Glc^+ and Gnt^+ mutants of R. sphaerodies. The appropriate strains were grown anaerobically in minimal medium with a 10 mM concentration of the indicated carbon source at 8,000-lux illumination. Symbols: ○, glucose, Glc^+ strain; ●, malate, Glc^+ strain; □, gluconate, Gnt^+ strain; ■, malate, Gnt^+ strain. (A) rate of H_2 evolution versus time; (B) total H_2 evolved versus time.*

growth and did not support H_2 production. The optimum initial NH_4Cl level both for growth of cells and for H_2 production was observed between 2 to 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 (Table 1). NH_4Cl at a concentration of 5 mM added to cultures producing gas completely suppressed H_2 production and inhibited CO_2 production. H_2 production was strongly influenced by the intensity of the incandescent light source. H_2 production increased at an approximately linear rate in the range of 1,000 to 12,000 lux, with the rate of production doubling with a doubling of illumination intensity, although intensities greater than 40,000 lux inhibited 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. The rate of H_2 production was linearly related to the cellular activity of nitrogenase (Fig. 5). This was true for each of the three carbon sources employed (malate, lactate, and glucose).

In a second series of experiments, nitrogen, acetylene, carbon monoxide, and oxygen were compared for their effects on H_2 production by cells limited for fixed nitrogen (Table 2). H_2 production from glucose was maximal under the inert atmosphere of Ar. As little as 5% acetylene in Ar was sufficient to diminish the H_2 evolution rate to less than one-half; 50% acetylene inhibited H_2 production completely. An atmosphere of 50% Ar–50% N_2 inhibited the acetylene reduction assay slightly, but lowered H_2 production to about 30% of the control level; 100% N_2 inhibited acetylene reduction by about two-thirds and suppressed H_2 production completely. Carbon monoxide was also added to cultures actively producing H_2 gas. 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.

Stoichiometry. Cultures of the glucose-utilizing strain (Glc^+), the gluconate-utilizing strain (Gnt^+), and of the wild type were assayed quantitatively (system ii) for substrate conversion by using radioactively labeled compounds (Table 3). The wild-type cells converted 24% of the available glucose to the equivalent amounts of H_2 (see equation 1). $^{14}CO_2$ recovered from the system was somewhat greater, consisting of 34% of the [^{14}C]glucose initially present. The non-

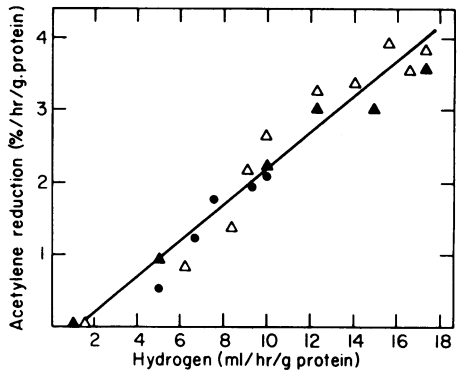

FIG. 5. *Comparison of H_2 evolution with acetylene reduction. Wild-type and Glc^+ mutants were grown anaerobically on minimal medium with a 20 mM concentration of the indicated carbon source in the light. %/hr/g protein: Acetylene reduction is presented as percent acetylene in atmosphere reacted per hour per gram of protein. Symbols: ▲, malate, wild-type strain; ▲, lactate, wild-type strain, ●, glucose, Glc^+ strain.*

TABLE 1. *Effect of ammonium on H_2 production*[a]

Time (h)	Concn of NH_4 in medium (mM)		OD_{640}[b]		Dry wt (mg/ml)		H_2 production (ml/h/g of protein)	
	A	B	A	B	A	B	A	B
0	2	6.1	50	50	0.073	0.076	0	0
10	0.4	1.5	130	140	0.091	0.089	0	0
14	<0.2	<0.2	180	220	0.110	0.109	0	1.0
18	<0.2	<0.2	190	240	0.112	0.107	0	13
22	—	<0.2	195	250	—	—	0.7	14
29	—	—	210	240	0.105	0.108	10	8
38	<0.2	<0.2	225	245	—	—	7.5	7
44	—	—	210	225	—	—	5	5
53	—	—	190	200	—	—	3	4
60	<0.2	<0.2	200	190	0.110	0.106	2	4

[a] Wild-type *R. sphaeroides* strains were grown anaerobically in the light with NH_4Cl as nitrogen source. Ammonia concentration was determined by Nessler's reagent. NH_4^+ could not be determined below 0.2 mM. —, Not determined.

[b] OD_{640}, Optical density, in Klett units, at 640 nm.

CO_2 radioactivity remaining was mostly in starting material [U-^{14}C]glucose, although on paper chromatography a compound with an R_f value 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 equation 2). With lactate as the carbon source, the wild type gave 48% conversion to H_2 and 58% of the theoretical CO_2. Although some (<5%) starting material remained for these two substrates, 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 by 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. Again, this is much higher than found in the wild type.

The possible identity of low-molecular-weight compounds formed during H_2 production was investigated by GLC analysis (Fig. 6). Spent culture-suspending media of the mutant Glc$^+$ and the wild type were trimethylsilylated and analyzed by GLC in these experiments. The Glc$^+$ mutant formed no discernible soluble product, and only the two isomeric forms of glucose were found at the completion of H_2 production. The wild-type organism 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".

TABLE 2. *Effect of inhibiting atmosphere on H_2 production*[a]

Atmosphere	Acetylene reduction[b]	H_2 production[c]
100% Ar	100	4.1
95% Ar-5% C_2H_2	—	1.7
50% Ar-50% C_2H_2	—	0.0
50% Ar-50% N_2	82	1.2
100% N_2	37	0.0
90% Ar-10% CO	<1	4.0
82% Ar-18% O_2	<0.5	0.0

[a] All cells were initially grown under Ar in minimal medium with 10 mM malate in the light. After the onset of visible gas production, cells 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.

[b] Values indicate relative percent C_2H_2 reduced per hour per gram of protein.

[c] Values indicate milliliters of H_2 evolved per hour per gram of protein.

DISCUSSION

Nitrogenase. The data in this paper support 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

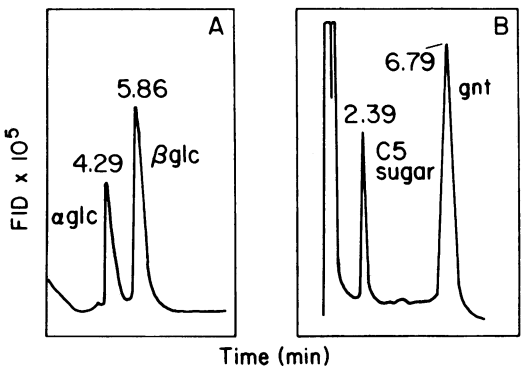

FIG. 6. *GLC analysis of TMS derivatives of culture media. After gassing, cultures were freeze-dried, trimethylsilylated, and separated by GLC, using a flame ionization detector (FID). (A) Glc$^+$ strain, (B) WT strain. αglc, TMS derivative of α-glucose; βglc, TMS derivative of β-glucose; gnt, TMS derivative of gluconic acid.*

TABLE 3. *Recovery of H_2 and CO_2 from gas-producing cultures*[a]

Cell type	Substrate	H_2 gas evolved	CO_2 gas eluted	Total label recovered
WT	Glucose, 0.5	1.4 (24)	1.0 (34)	(91)
WT	Malate, 0.5	1.7 (57)	1.25 (60)	(92)
WT	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] All cultures were grown in minimal medium with 0.2% yeast extract as nitrogen source and 10 mM carbon source (pH 7.2). Other conditions were as described in the text. Values indicate, in millimoles, averages of four trials, with analysis done after cessation of measurable gas evolution. Percent values, in parentheses, are of theoretical maximum yields, assuming complete conversion. WT, Wild-type *R. sphaeroides*; Glc$^+$, glucose-utilizing mutant; Gnt$^+$, gluconate-utilizing mutant.

substrates for reduction catalyzed by nitrogenase, inhibited H_2 evolution. Second, the ammonium ion, which is generally a repressor of nitrogenase synthesis (15), 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 on in situ H_2 biosynthesis were predictable, based on published results with the purified enzyme (9). As reported by others, CO blocks nitrogenase-catalyzed reduction of molecular nitrogen without inhibiting H_2 evolution (8, 9). We have demonstrated a similar effect for *R. sphaeroides*.

Mutants. 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-h) lag period. However, selection of the Glc^+ and Gnt^+ 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 strains were stable when maintained on a nonspecific (rich) medium without the appropriate carbon compound. They would produce H_2 when returned to minimal conditions and the carbon compound with only the usual lag to deplete the fixed nitrogen in the system. 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.

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.

LITERATURE CITED

1. **Anderson, L., and R. C. Fuller.** 1967. Photosynthesis in *Rhodospirillum rubrum*. III. Metabolic control of the reductive pentose phosphate and TCA cycle enzymes. Plant Physiol. **42**:497–502.
2. **Cohen-Bazire, G., W. R. Sistrom, and R. Y. Stanier.** 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. J. Cell. Comp. Physiol. **49**:25–68.
3. **Conrad, R., and H. G. Schlegel.** 1977. Influence of aerobic and phototrophic growth conditions on the distribution of glucose and fructose carbon into the Entner-Doudoroff and Embden-Meyerhof pathways in *Rhodopseudomonas sphaeroides*. J. Gen. Microbiol. **101**: 277–290.
4. **Eisenberg, M. A.** 1953. The tricarboxylic acid cycle in *Rhodospirillum rubrum*, J. Biol. Chem. **203**:815–836.
5. **Gest, H., and M. D. Kamen.** 1949. Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*. Science **109**:558–559.
6. **Gest H.** 1951. Metabolic patterns in photosynthetic bacteria. Bacteriol. Rev. **15**:183–210.
7. **Gest, H., J. G. Ormerod, and K. I. Ormerod.** 1962. Photometabolism of *Rhodospirillum rubrum*: light-dependent dissimilation of organic compounds to CO_2 and molecular H_2 by an anaerobic citric acid cycle. Arch. Biochem. Biophys. **97**:21–33.
8. **Hillmer, P., and H. Gest.** 1977. Hydrogen metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: production and utilization of H_2 by resting cells. J. Bacteriol. **129**:732–739.
9. **Hwang, J. C., C. H. Chen, and R. H. Burris.** 1973. Inhibition of nitrogenase-catalyzed reductions. Biochim. Biophys. Acta **292**:256–270.
10. **Kanazawa, T., M. R. Kirk, and J. A. Bassham.** 1970. Regulatory effects of ammonia on carbon metabolism in photosynthesizing *Chlorella pyrenoidosa*. Biochim. Biophys. Acta **205**:401–408.
11. **Koch, B., and H. J. Evans.** 1966. Reduction of acetylene to ethylene by soy bean root nodules. Plant Physiol. **41**: 1748–1752.
12. **Laine, R. A., W. J. Esselman, and C. C. Sweeley.** 1972. Gas-liquid chromatography of carbohydrates. Methods Enzymol. **28**:159–967.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and A. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **McNary, J. E., and R. H. Burris.** 1962. Energy requirements for nitrogen fixation by cell-free preparations from *Clostridium pasteurianum*. J. Bacteriol. **84**:598–599.
15. **Ormerod, J. G., K. S. Omerod, and H. Gest.** 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94**:449–463.
16. **Pedersen, T. A., M. R. Kirk, and J. A. Bassham.** 1966. Light-dark transients in levels of intermediate compounds during photosynthetics in air-adapted *Chlorella*. Physiol. Plant. **19**:219–231.
17. **Szymona, M., and M. Doudoroff.** 1960. Carbohydrate metabolism in *Rhodopseudomonas spheroides*. J. Gen. Microbiol. **22**:167–183.
18. **Watt, G. D., W. A. Bulen, A. Burns, and K. L. Hadfield.** 1975. Stoichiometry, ATP/2e values and energy requirements for reactions catalyzed by nitrogenase from *Azotobacter vinelandii*. Biochemistry **14**:4266–4272.
19. **Yagi, T.** 1970. Solubilization, purification and properties of particulate hydrogenase from *Desulfovibrio vulgaris*. J. Biochem. **68**:649–657.