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H_2 Metabolism in the Photosynthetic Bacterium *Rhodopseudomonas capsulata*: H_2 Production by Growing Cultures

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Purple photosynthetic bacteria produce H_2 from organic compounds by an anaerobic light-dependent electron transfer process in which nitrogenase functions as the terminal catalyst. It has been established that the H_2-evolving function of nitrogenase is inhibited by N_2 and ammonium salts, and is maximally expressed in cells growing photoheterotrophically with certain amino acids as sources of nitrogen. In the present studies with *Rhodopseudomonas capsulata*, nutritional factors affecting the rate and magnitude of H_2 photoproduction in cultures growing with amino acid nitrogen sources were examined. The highest H_2 yields and rates of formation were observed with the organic acids: lactate, pyruvate, malate, and succinate in media containing glutamate as the N source; under optimal conditions with excess lactate, H_2 was produced at rates of ca. 130 ml/h per g (dry weight) of cells. Hydrogen production is significantly influenced by the N/C ratio in the growth substrates; when this ratio exceeds a critical value, free ammonia appears in the medium and H_2 is not evolved. In the "standard" lactate + glutamate system, both H_2 production and growth are "saturated" at a light intensity of ca. 600 ft-c (6,500 lux). Evolution of H_2, however, occurs during growth at light intensities as low as 50 to 100 ft-c (540 to 1,080 lux), i.e., under conditions of energy limitation. In circumstances in which energy conversion rate and supplies of reducing power exceed the capacity of the biosynthetic machinery, energy-dependent H_2 production presumably represents a regulatory device that facilitates "energy-idling." It appears that even when light intensity (energy) is limiting, a significant fraction of the available reducing power and adenosine 5'-triphosphate is diverted to nitrogenase, resulting in H_2 formation and a bioenergetic burden to the cell.

Light-dependent production of molecular hydrogen by photosynthetic bacteria was first observed in cultures of *Rhodospirillum rubrum* growing photoheterotrophically (anaerobically) in media containing dicarboxylic acids of the citric acid cycle and either glutamate or aspartate as nitrogen sources (5, 6). Hydrogen is not evolved, however, when nitrogen is provided by ammonium salts, which repress synthesis of the hydrogen-evolving system (17). Illuminated resting cells of *R. rubrum*, derived from cultures producing hydrogen, evolve large quantities of H_2 from a variety of organic compounds, and this process is quickly and severely inhibited upon addition of an ammonium salt or molecular nitrogen (7). These findings made it apparent that photoproduction of H_2 is intimately connected with nitrogen metabolism, and led to the discovery that *R. rubrum* is capable of nitrogen fixation (7, 12). Similar observations were made over the course of the past 25 years with a variety of photosynthetic bacteria (e.g., see [2, 9, 15, 19]), but quantitative studies of the factors affecting H_2 evolution by growing cultures have not been published. In this communication, we describe relevant experiments with the nonsulfur purple bacterium *Rhodopseudomonas capsulata*.

MATERIALS AND METHODS

Bacterial strains. *R. capsulata* Z-1 is an arsenate-resistant mutant of wild-type strain St. Louis (ATCC 23782) (24). Strain J2 was derived from wild-type strain B10 (14), which carries two kinds of bacteriophages (22); strain J2 is a spontaneous rifampin-resistant mutant of B100, which is a spontaneous mutant of B10 cured of phages. Strain L_1 is a "gain of function" mutant (of strain St. Louis) that has acquired the capacity to use glycerol as a carbon source for growth (13).

Growth media. All media contained mineral

salts, thiamine (a required growth factor), and ethylenediaminetetraacetic acid as specified by Weaver et al. (23) for medium RCV. (Note that the recipe given in reference 23 inadvertantly omitted 12 mg of $FeSO_4 \cdot 7H_2O$ per liter.) For growth of "precultures" and for many experiments, a standard medium was used, consisting of the basal medium supplemented with 30 mM DL-lactate and 7 mM L-glutamate; other concentrations of lactate and glutamate were used as noted. Other variations in nature and concentrations of carbon and nitrogen sources are detailed in connection with the experimental results. All amino acids used were L isomers, and their solutions were sterilized by filtration.

Precultures. Precultures, to provide inocula for experimental cultures, were grown anaerobically in completely full test tubes of 17-ml capacity, closed with solid rubber stoppers (to permit gas escape, the stopper was fitted with a narrow-gauge hypodermic needle). The tubes were incubated at 30°C with illumination from Lumiline incandescent lamps (ca. 600 ft-c [6,500 lux]), and the cultures were transferred daily. At the time of inoculation of experimental cultures, precultures had been grown for at least 10 generations and were not older than 17 h.

Growth of experimental cultures: measurement of H_2 production. All cultures were grown photosynthetically under anaerobic conditions. For most experiments, modified glass syringes (nominal capacity, 30 ml) served as the growth vessels; the usual external end of the hollow plunger is cut off and the plunger is inverted. This provides a device in which gas produced by the culture (within the syringe body and plunger) accumulates in the plunger, displacing it upwards. The plunger is precalibrated by injection of known quantities of air, and the volume of accumulated gas is directly indicated. During the syringe assembly procedure, medium and inoculum are continuously gassed with a stream of argon bubbles to maintain anaerobiosis. The syringe is completely filled with inoculated medium (60 ml, unless otherwise noted), residual gas is expelled through the syringe needle, and the syringe needle is then stabbed into a solid rubber stopper. Syringes prepared in this way were incubated in a glass-sided water bath maintained at 35°C and illuminated (ca. 1,000 ft-c [10,800-lux] intensity, except as noted) by an external bank of Lumiline lamps. As growth proceeds, samples of suspension for determination of bacterial density, etc., can be readily removed through the syringe needle and, as necessary, gas can be similarly expelled. Figure 1 shows a set of four syringes after 7 h of illumination, at which time ca. 20 ml of H_2 had been produced in lactate + glutamate medium (no. 1). Although growth was heavy in syringes 2 and 3, which contained media supplemented with ammonium sulfate, no H_2 was evolved; the relatively small quantity of H_2 seen with glutamate alone (no. 4) was due to residual lactate present in the inoculum.

With organic acids (such as lactate and malate) serving as the primary sources of cell carbon, the pH rises during growth to the extent that practically all of the metabolic CO_2 produced is bound, and the accumulated gas consequently consists mainly of

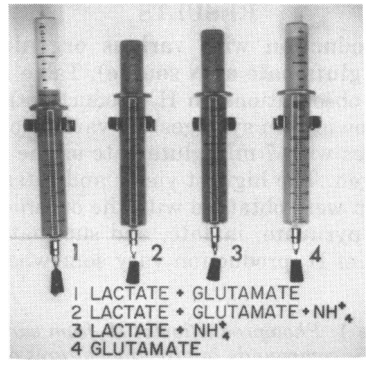

FIG. 1. *Syringe technique (devised by P. F. Weaver) for measuring H_2 production by growing cultures of purple bacteria; effect of NH_4^+ on H_2 formation. For this experiment, the basal medium was supplemented with 0.07% yeast extract (Difco) and 0.07% peptone (Difco) (to accelerate growth) and, where indicated: DL-lactate, L-glutamate, and $(NH_4)_2SO_4$ at concentrations of 30 mM, 0.1, and 0.1%, respectively. At zero time, each syringe was completely full of inoculated medium (60-ml total volume, including 7 ml of a 17-h-old photosynthetically grown culture [lactate + glutamate medium] of R. capsulata strain Z-1). The syringes were illuminated (at 35°C) and formation of bubbles of H_2 was evident in syringe 1 (lactate + glutamate medium) within 2 h; the photograph was taken after 7 h of incubation.*

H_2. This was checked in control experiments by introducing strong alkali to absorb gaseous CO_2. In an extreme situation with a large excess of lactate relative to glutamate, CO_2 appeared in the gas phase starting at ca. 15 h of incubation but constituted less than 15% of the total gas produced from the complete dissimilation of the lactate; since this can be considered an upper limit, not reached in most experiments, gas volumes are given as "H_2 produced" without correction.

Analytical determinations. Glutamate and lactate concentrations were determined enzymatically using glutamate (1) and lactate (11) dehydrogenases, respectively.

Ammonia concentrations were estimated with an ammonia electrode purchased from Orion Research, Inc., Cambridge, Mass. Samples of bacterial suspension (6 to 10 ml) were centrifuged, and the supernatant fluids (room temperature) were supplemented with 0.05 to 0.1 ml of 10 N NaOH; the electrode was immediately immersed into the alkalinized sample, and readings were taken after 5 min of stirring. Over the range employed, standard curves (using ammonium sulfate) were linear down to 10 μM NH_4^+.

Other determinations. Bacterial concentrations were measured either by determining culture absorbancy at 660 nm (Zeiss PMQ2 spectrophotometer) or dry weight of centrifuged cells (washed once with distilled water and dried in preweighed aluminum cups at 90°C for 24 h).

RESULTS

H_2 production with various organic substrates (glutamate as N source). Table 1 summarizes observations on H_2 production by cultures growing (in syringes) on various organic substrates with 7 mM glutamate as the source of nitrogen. The highest yields and rates of H_2 evolution were obtained with the organic acids lactate, pyruvate, malate, and succinate. The kinetics of H_2 production vary somewhat with different carbon sources, presumably because of differences in their reduction states and patterns of metabolism. With the strain of *R. capsulata* used, lactate was consistently the best substrate in respect to vigor of H_2 formation. Moreover, the ca. 72% yield of H_2 observed with lactate suggests that, under certain conditions, this substrate can be completely dissimilated to $H_2 + CO_2$; resting cells of *R. rubrum*, in fact, are capable of catalyzing such conversions by means of an anaerobic citric acid cycle (8).

TABLE 1. *Photoproduction of H_2 from various organic compounds in growing cultures of R. capsulata*[a]

Substrate	Hydrogen production	
	Rate[b] (μl/h per mg [dry wt] of cells)	Approx yield (% of theoretical maximum[c])
D-Glucose	88	32
D-Fructose	100	27
Sucrose	60	6
DL-Lactate	130	72
Pyruvate	130	68
DL-Malate	90	56
Succinate	100	72
Propionate	40	ND[d]
Butyrate	20	ND

[a] Cultures of *R. capsulata* Z-1 were grown in syringes as described in Materials and Methods, using media containing 30 mM substrate and 7mM L-glutamate; in each instance, the inoculum was from a preculture grown on the same substrate. Initial bacterial concentrations in the experimental cultures were ca. 0.4 absorbancy unit at 660 nm.

[b] Rates were calculated from data obtained after exhaustion of the N source.

[c] For complete dissimilation of the original quantity of substrate to $H_2 + CO_2$.

[d] ND, Not determined.

It should be noted that when the N source is an ammonium salt (and the H_2 evolution system is repressed), anaerobic growth of photosynthetic bacteria on reduced C sources such as propionate and butyrate occurs only if substrate quantities of CO_2 are also supplied (see discussion in reference 16). On the other hand, with glutamate as N source, H_2 evolution occurs and CO_2 addition is unnecessary. In other words, when H_2 production is possible, CO_2 is no longer required as an electron "sink." The same considerations apply to glycerol as a C source; in separate experiments comparable to those of Table 1, it was found that *R. capsulata* strain L_1 grows readily on glycerol + glutamate (without CO_2 addition) and produces H_2 at a rate of ca. 40 μl/h per mg (dry weight) of cells.

H_2 production with lactate and various amino acid nitrogen sources. Nineteen amino acids were examined as N sources in special regard to rate of H_2 evolution with lactate as the primary C source. The results presented in Table 2 show that the amino acids can be divided into two groups in respect to efficacy in supporting growth and H_2 formation. These were best with amino acids grouped on the left (glutamate, etc.), which gave doubling times varying from 2.1 to 3.9 h and rates of H_2 evolu-

TABLE 2. *Photoproduction of H_2 and growth rates of R. capsulata in media containing 30 mM DL-lactate and various amino acids as N sources*[a]

Amino acid[b]	Rate of H_2 production (μl/h per ml of culture)	Doubling time (h)	Amino acid[b]	Rate of H_2 production (μl/h per ml of culture)	Doubling time (h)
Glutamate	130	3.0	Leucine	99	5.6
Serine	130	3.1	Valine	76	11.0
Alanine	130	3.2	Isoleucine	68	6.4
Proline	120	3.1	Phenylalanine	55	4.3
Ornithine	120	3.9	Tryptophan	52	6.1
Tyrosine	115	3.3	Methionine	38	16.0
Glutamine	110	2.4	Threonine	30	6.7
Aspartate	100	2.9	Lysine	6	19.0
Asparagine	100	2.1	Cysteine	<1	6.0
Arginine	100	2.1			

[a] Experimental conditions as in Table 1, except that the inocula were from precultures grown in 30 mM DL-lactate + 7 mM L-glutamate medium.

[b] Amino acids were added at 7 mM concentration.

tion from 100 to 130 μl/h per ml of culture. In contrast to our results with *R. capsulata*, Bregoff and Kamen (3) observed that with *R. rubrum* a group of nine amino acids (including serine, alanine, glutamine, and asparagine) supported growth with malate but not H_2 formation; in these instances, a "steady-state concentration" of free ammonia was detected in the cultures.

On the basis of the results already presented, lactate + glutamate was selected as a "standard system" for more detailed study.

Standard lactate + glutamate system. The effects of varying the initial concentrations of lactate and glutamate in the growth medium are shown in Fig. 2. Hydrogen production occurs throughout the growth period, and this is more clearly seen when the growth phase is extended by addition of glutamate at high concentration (22 mM). Increase of glutamate concentration from 7 to 22 mM did not alter growth rate significantly but, of course, resulted in higher final cell yields; thus, with 40 mM lactate, typical cell yields for 7, 16, and 22 mM glutamate were 1.3, 2.6, and 3.3 mg (dry weight) of cells per ml, respectively. After exhaustion of glutamate, the cells enter the stationary phase and continue to dissimilate remaining lactate to H_2 + CO_2, until all of the substrate has disappeared. Obviously, the fraction of the lactate used for biosynthesis (and, consequently, not converted to H_2 + CO_2) is related to the quantity of N source available, and this influences the total gas yield. At the extreme with 40 mM lactate, as the glutamate concentration increases from 7 to 22 mM, there is a progressive decrease in both H_2 formation rate and yield; with low lactate concentration (6 mM), all of the substrate is utilized for biosynthesis before glutamate is exhausted, and H_2

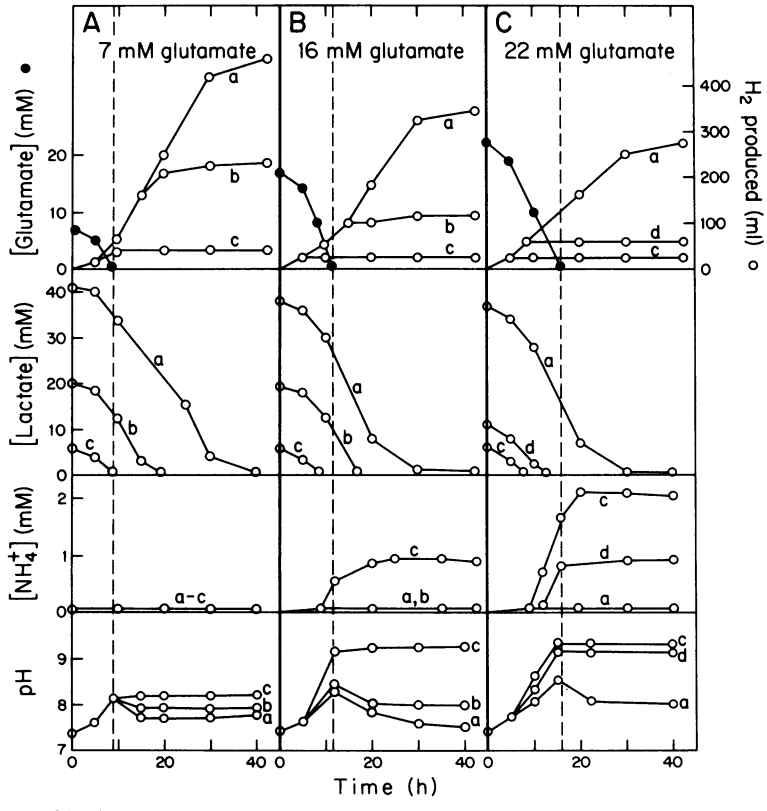

FIG. 2. *Kinetics of H_2 formation and substrate utilization during photosynthetic growth of R. capsulata J2 in media containing various ratios of glutamate/lactate; changes in pH and NH_4^+ are also shown. Large syringes, each initially containing 100 ml of inoculated medium, were used; inocula: from precultures grown in 30 mM* DL-*lactate + 7 mM* L-*glutamate medium, sufficient to give an initial bacterial concentration of 0.3 to 0.4 absorbancy unit at 660 nm. Lactate, glutamate, and ammonia concentrations were determined as noted in Materials and Methods. The vertical dashed lines indicate the times of glutamate exhaustion. Initial glutamate concentrations: (A) 7 mM, (B) 17 mM, (C) 22 mM. Initial lactate concentrations: (a) 40 mM, (b) 20 mM (c) 6 mM, (d) 12 mM.*

production is, therefore, negligible. The effect of increasing glutamate concentration on rate of H_2 formation is probably due, in part at least, to markedly increased "self-shading" that occurs as the cell concentration rises (i.e., average light intensity in the interior of the culture progressively decreases).

When the amount of lactate available is relatively small, a proportionately greater fraction is used for cell biosynthesis and, consequently, the relationship between total H_2 production and initial lactate concentration is not linear; this is shown in Fig. 3 for the three glutamate concentrations used in the experiments of Fig. 2.

Figure 2 shows the pH changes in cultures growing with various lactate and glutamate concentrations. In all instances, the pH rises during the growth period due to accumulation of alkali as a consequence of consumption of organic acid anions; when lactate is in excess, however, the pH shows a secondary decrease, which possibly is due to production of an acidic product.

Ammonia production with excess glutamate. If lactate is exhausted earlier than glutamate, the remaining amino acid is partially deaminated and NH_4^+ becomes detectable in the medium (Fig. 2B and C). Under such circumstances, H_2 is not produced in significant quantity. The initial ratio of glutamate to lactate determines whether or not net ammonia is produced, and this is further illustrated by the

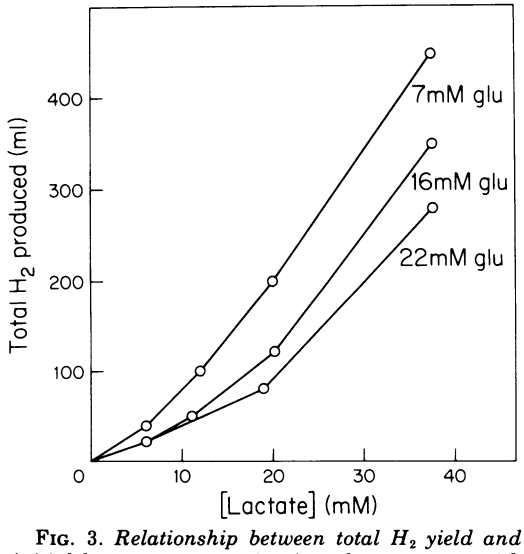

FIG. 3. *Relationship between total H_2 yield and initial lactate concentration in cultures grown with several different initial glutamate concentrations. Data from Fig. 2 were replotted to show the relationship.*

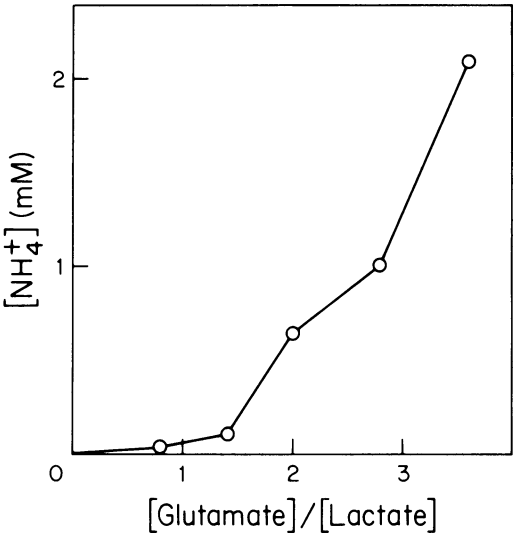

FIG. 4. *Ammonia formation by cultures of R. capsulata J2 containing different initial ratios of glutamate/lactate. The data are from Fig. 1 and additional experiments of the same kind. Final NH_4^+ concentrations were determined using an ammonia electrode.*

data of Fig. 4. Thus, when the glutamate/lactate ratio exceeds ca. 1, ammonia appears in the medium after the lactate has been consumed; and the NH_4^+ level increases as the glutamate/lactate ratio increases.

Amino acids as sole C and N sources. A survey of amino acids disclosed that those listed in Table 3 individually support anaerobic photosynthetic growth of *R. capsulata* as sole sources of both C and N. In all instances, ammonia was produced during growth and H_2 evolution did not occur. Other experiments showed that the quantity of NH_4^+ produced is proportional to the initial concentration of such amino acids (up to 15 mM), and is related to the N/C ratio in the amino acid. The latter is indicated by the data of Fig. 5, for three amino acids with different N/C ratios. It can be seen that ammonia production increases with increasing N/C ratio in the amino acid substrate. Assuming that net ammonia is not produced with amino acids having the same N/C ratio as cell material, the latter can be estimated from such experiments; these indicated a value of ca. 0.18 for *R. capsulata*, in good agreement with values in the literature (20) of about 0.2 for various photosynthetic bacteria.

Effect of light intensity on growth and H_2 production. Rates of growth and H_2 production in 30 mM lactate + 7 mM glutamate medium as a function of light intensity are depicted in Fig. 6. Both become "saturated" at an intensity of

TABLE 3. *Ammonia production and growth rates of R. capsulata J2 in media containing single amino acids as sole sources of C and N*[a]

Amino acid	Final NH_4^+ concn (mM)	Doubling time (h)
Arginine	27.0	4.2
Asparagine	24.0	4.7
Glutamine	10.5	4.5
Serine	8.1	6.3
Alanine	8.0	2.8
Aspartate	6.5	5.6
Glutamate	2.9	2.9

[a] For these experiments, the basal medium was supplemented with individual amino acids at 14 mM concentration. The cultures were grown in completely full screw-cap test tubes; inocula, as in Table 2. NH_4^+ concentrations were measured with an ammonia electrode after growth had ceased.

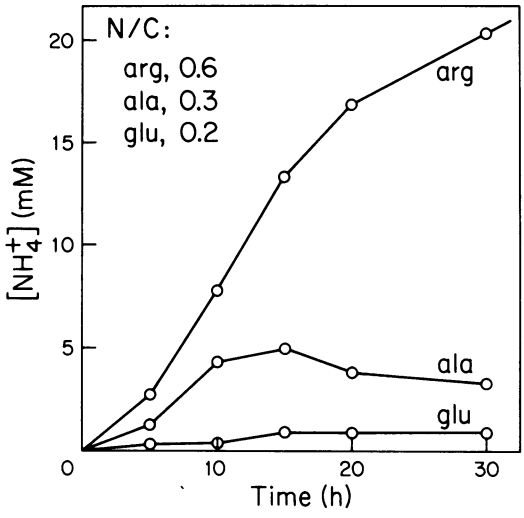

FIG. 5. *Kinetics of ammonia formation during growth of R. capsulata J2 on single amino acids as sole sources of both C and N. Basal medium was supplemented with the individual amino acids at 14 mM concentration; inocula, as in Fig. 2. arg, Arginine; ala, alanine; glu, glutamate. Note that the exact N/C ratios for alanine and arginine are 0.33 and 0.67, respectively.*

ca. 600 ft-c (6,500 lux). The saturation point for growth presumably reflects the maximal rate of photophosphorylation attainable under the conditions used, and it is understandable that the energy-dependent production of H_2 also saturates at the same light intensity. It is of particular significance that H_2 evolution occurs throughout the range where light intensity limits its growth rate. Thus, H_2 formation is detectable even at light intensities of 50 to 100 ft-c (540 to 1,080 lux), i.e., at very low growth rates. These observations imply that, in wild-type cells under conditions in which the H_2-evolving system is not repressed, energy-dependent electron flow occurs, resulting in H_2 formation even when the cells are under energy stress. In other words, H_2 evolution may in some circumstances represent a bioenergetic burden. This interpretation has significant implications for the physiological roles of photoproduction of H_2, which are considered further in the Discussion.

DISCUSSION

Our understanding of the mechanism and significance of light-dependent H_2 formation by photosynthetic bacteria stems primarily from earlier studies with *R. rubrum* (reviewed in reference 4). These researches indicated that H_2 evolution occurs only under conditions where nitrogenase synthesis is not repressed, and more recent investigations with *R. capsulata* (21) have provided conclusive evidence that the in vivo formation of H_2 is catalyzed by the nitrogenase complex. Photoproduction of H_2 from organic substrates is inhibited by the addition of N_2 or NH_4^+ (7), both of which are readily used for reductive biosynthesis of amino acids and other cellular constituents; in addition to causing repression of synthesis of "nitrogenase-hydrogenase," NH_4^+ is a potent inhibitor of N_2 reduction per se by photosynthetic bacteria (18). In the presence of these N sources, the H_2-evolving function of nitrogenase is inhibited; evidently, reducing power and adenosine 5'-triphosphate (ATP) in these circumstances are directed toward biosynthesis rather than being diverted for the production of H_2. In both *R. rubrum* and *R. capsulata*, maxi-

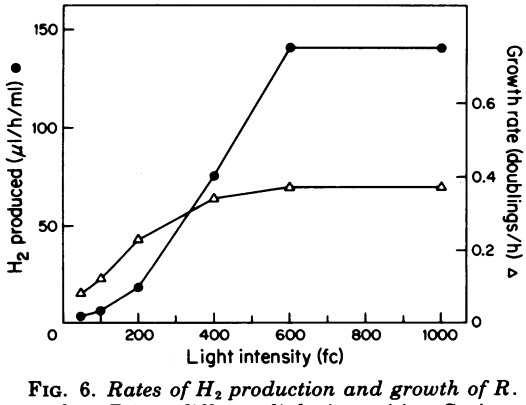

FIG. 6. *Rates of H_2 production and growth of R. capsulata Z-1 at different light intensities. Syringe cultures (30 mM* DL-*lactate + 7 mM* L-*glutamate medium) were incubated at the various light intensities indicated. Symbols: (●) Rate of H_2 formation, (△) growth rate.*

mal expression of H_2 evolution capacity is observed when the organisms are grown photoheterotrophically on organic acids with certain amino acids serving as sources of nitrogen. The present studies demonstrate that, under such conditions, the rate and extent of H_2 formation are dictated by the light intensity and quantity of non-nitrogenous organic substrate available in excess of biosynthetic requirements. The latter is, of course, related to the amount of amino acid N available, which in effect means that the N/C ratio in the organic nutrients provided is of relevance.

From the results described it is also clear that the steady-state concentration of free ammonia produced in cultures growing with amino acid N sources is of critical importance in respect to H_2 formation. Ammonia, even at very low concentrations, inhibits H_2 production and, from experiments reported in the succeeding paper (10), it can be identified as a prominent regulator of H_2 metabolism in *R. capsulata*. In lactate + glutamate media, the formation of free ammonia occurs when the glutamate/lactate ratio exceeds ca. 1, and the relevance of N/C ratio in the growth substrates for net ammonia formation is particularly evident when amino acids are used as the sole sources of both N and C.

Maximal photoproduction of H_2 by purple bacteria is observed under conditions in which cells presumably regenerate ATP and reduced pyridine nucleotides in great excess of the demands of the biosynthetic apparatus (illuminated resting cells supplied with readily utilizable organic substrates such as lactate, pyruvate, or dicarboxylic acids [4, 8]). Accordingly, the energy-dependent formation of H_2 has been interpreted as a control device that aids in adjusting the levels of reducing power and ATP to values appropriate to the intensity of biosynthetic activity (4). Looked at in this way, H_2 formation is seen as a fine-tuning mechanism for energy-idling when this is required by the balance between energy conversion activity and overall biosynthetic rate. The experiments reported on H_2 production as a function of light intensity, however, indicate that H_2 formation also occurs in growing cultures under conditions where growth rate is limited by the supply of radiant energy, i.e., when the ATP supply is limiting, rather than excessive. The hydrogen (H_2) production activity of nitrogenase observed during growth under circumstances where the enzyme complex is not functioning to provide cell nitrogen can be interpreted as a leak of energy and reducing power that results from unregulated energy-dependent electron flow to nitrogenase. When growth rate is limited by light intensity, it would seem that the occurrence of H_2 evolution must impose a bioenergetic burden, that is, light-dependent formation of H_2 can represent a dissipation of energy that might otherwise be utilizable for biosynthesis. The magnitude of the burden under such conditions remains to be determined.

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