STUDIES ON THE METABOLISM OF PHOTOSYNTHETIC BACTERIA

IV. Photochemical Production of Molecular Hydrogen by Growing Cultures of Photosynthetic Bacteria

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The outstanding characteristic of nonsulfur purple bacteria (*Athiorhodaceae*) is the ability to reduce CO_2 photochemically in the presence of organic hydrogen donors (Gaffron, 1933, 1935). These organisms differ from the sulfur purple bacteria (*Thiorhodaceae*) in two major respects: (a) they are unable to use reduced sulfur compounds as hydrogen donors, and (b) they require preformed vitamins for growth (Hutner, 1944, 1946). Although some species of *Athiorhodaceae* tolerate the presence of oxygen or may even develop aerobically in the dark to some extent, growth is generally optimal under anaerobic conditions in the light (van Niel, 1944). In the absence of oxygen, growth occurs only if the cultures are illuminated. Under these circumstances, purple bacteria are remarkably efficient as compared with typical heterotrophic anaerobes; thus far no significant quantity of any metabolic product other than CO_2 has been found in the medium of luxuriant cultures grown on "physiological" substrates (Gaffron, 1933; Muller, 1933).

Muller (1933) reported that anaerobic growth of sulfur purple bacteria in media containing mineral salts and organic compounds more oxidized than carbohydrate was accompanied by a net production of CO_2. On the other hand, when using more reduced organic hydrogen donors, he found it necessary to supply CO_2 in order to obtain growth. These observations on the growth of sulfur purple bacteria appear to be descriptive for the growth of *Athiorhodaceae* also.

Except for CO_2, no other gaseous products of *photosynthetic* activity have been noted previously in purple bacteria. The anaerobic production of H_2 by resting cells of a sulfur purple bacterium grown in peptone media was described by Roelofsen (1935). In this case, H_2 evolution was observed only during a dark "autofermentation" and was considered an artifact associated with autolysis of the cells. It was later shown by Nakamura (1937, 1939) that resting cells of *Rhodobacillus palustris* (*Athiorhodaceae*) and *Chromatium minutissimum* (*Thiorhodaceae*) decompose formate, glucose, pyruvate, glycerol, and glycerophosphate anaerobically in the dark with the production of H_2 as an end product.

In the present paper, the formation of H_2 (and CO_2) in growing cultures of *Rhodospirillum rubrum* (*Athiorhodaceae*) is described. Hydrogen is produced by this organism during growth on certain oxidized substrates in synthetic or semi-

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synthetic media under anaerobic conditions in the light. Experiments with resting cells derived from such cultures have demonstrated unequivocally that the H_2 evolution is a light-dependent reaction and that the yield of H_2 can exceed one mol per mol of substrate added (Gest and Kamen, 1949; Gest, Kamen, and Bregoff, 1949). The phenomenon is unusual and unexpected because of the simultaneous production of excess CO_2, which ordinarily is capable of acting as a hydrogen acceptor.

EXPERIMENTAL PROCEDURES AND RESULTS

Hydrogen production in synthetic media. The production of H_2 by growing cultures of *Rhodospirillum rubrum* (strain SI) was originally noted in the following medium: fumaric acid, 3 g (or DL-malic acid, 3.5 g); L-glutamic acid, 4 g; potassium citrate·H_2O, 0.8 g; biotin 25 μg; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 38 mg; KH_2PO_4, 16 mg; K_2HPO_4, 24 mg; distilled water, 1 liter.[2] The pH was adjusted to 7 with NaOH before autoclaving. Anaerobic cultures were prepared by completely filling small glass-stoppered reagent bottles with inoculated medium. No special precautions were taken to remove air dissolved in the medium before inoculation. The bottles were illuminated with incandescent lamps at a temperature of approximately 30 C and the cultures shaken once a day to disperse sedimented bacteria.

The formation of CO_2 and H_2 was manifested by the appearance of a considerable gas space at the top of the bottle after displacement of medium around the glass stopper; it was necessary to tape the stopper down, since the gas production was ordinarily sufficiently vigorous to expel it from the bottle. Identification of H_2 in the gas was readily accomplished by explosion with palladinized asbestos, prepared according to the directions of Treadwell and Hall (1928). The production of an alkali-insoluble gas that is combustible with air in the presence of palladinized asbestos can also be demonstrated using Smith fermentation tubes in the usual manner.

The possibility that a nonphotosynthetic hydrogen-producing contaminant was responsible for gas evolution was eliminated by the following evidence: (a) no organisms other than the characteristic spirillae could be observed microscopically in the cultures, (b) no growth or gas production occurred if the inoculated cultures were incubated in the dark, and (c) illuminated yeast extract agar shake cultures made from bottles showing gas production contained colonies of purple bacteria only.

General nutritional requirements. Biotin has recently been identified as the single *essential* organic growth factor for various strains of *R. rubrum* (Hutner, 1944, 1946). We have observed, however, that the addition of yeast extract (250 mg Difco yeast extract per liter) to the synthetic media invariably leads to much more rapid growth and consequently to an earlier appearance of H_2 and CO_2 in cultures of *R. rubrum* (SI). From the results of growth experiments with the foregoing and numerous other media with various organic hydrogen donors, it

[2] This medium is a modification of a recipe suggested by Dr. S. H. Hutner in a private communication.

appears that yeast extract supplies as yet unknown compounds that are required in addition to biotin for optimal growth. The results obtained also suggest that the requirements for optimal growth may vary, depending on the nature of the carbon and nitrogen sources provided.

The standard procedure now used in this laboratory for obtaining active hydrogen-producing bacteria for resting cell experiments is as follows: A medium of the composition given below is sown with a generous inoculum from a stab culture in 1 to 2 per cent agar plus 0.3 per cent Difco yeast extract.[3] Composition of liquid medium: DL-malic acid, 3.5 g; L-glutamic acid, 4 g; sodium citrate·$5\frac{1}{2}H_2O$, 0.8 g; biotin, 5μg; $MgSO_4 \cdot 7H_20$, 0.2 g; $CaCl_2$, 38 mg; KH_2PO_4, 120 mg; K_2HPO_4, 180 mg; Difco yeast extract, 250 mg; distilled water, 1 liter; pH adjusted to 7 with NaOH before autoclaving. With a stab inoculum as the starter, excellent growth is usually obtained in 3 to 4 days under anaerobic conditions in the light (at a temperature of about 30 C).[4] The use of a large liquid inoculum (>0.5 per cent) from peptone or yeast extract cultures is not recommended because of the inhibitory effect of these complex substances on H_2 formation.

It is of interest to note that the amount of phosphate required by *R. rubrum* (SI) for maximal growth is considerably less than that normally used in culture media. Separate experiments with several synthetic media have shown that the cell yields do not fall off appreciably until the phosphate level is reduced to less than 2 to 4 mg P per liter.

Substrates necessary for H_2 production. In an attempt to determine which organic components of the medium were required for H_2 evolution, growth experiments were conducted using media containing the individual substrates and the various possible combinations. Ammonium chloride (1 g per liter) was added in cases in which glutamic acid was omitted. Culture experiments of this kind indicated that H_2 was formed only if glutamic and fumaric acids were both present. The results were unaffected by the presence or absence of citrate, which was added simply as a complexing agent to prevent precipitation of insoluble compounds.[5]

R. rubrum (SI) grows equally well and produces H_2 in media of the composition already given but containing succinic or malic acids in place of fumarate. Preliminary experiments also indicate that aspartic acid may be substituted for glutamate as a nitrogen source; it is possible that other amino acids will be found to be suitable.

Growth experiments using a "basal" glutamate-malate medium supplemented with different quantities of Difco yeast extract disclosed that H_2 formation is not observable when the concentration of yeast extract is 1 gram per liter or higher. Similarly, addition of NH_4Cl (0.5 to 2 g per liter) or Difco peptone (10 g per liter)

[3] Freshly prepared medium of this composition is inoculated and incubated in the light to provide stock stab cultures. Good growth is obtained in 5 to 6 days.

[4] Moderately good growth is obtained under "semiaerobic" conditions also. Numerous other strains of *R. rubrum* will grow well and produce H_2 anaerobically in this medium.

[5] Citrate is not readily utilizable by *R. rubrum* (SI) as a carbon source for growth. It is not metabolized appreciably by resting cells under anaerobic conditions in the light.

to the synthetic medium abolishes hydrogen production. The inhibitory substances present in the yeast extract and peptone are as yet unknown. In connection with the effect of these materials, it is of interest that Gunsalus (1947) has reported the presence of unknown hydrogen acceptors in yeast extract which participate in the anaerobic fermentation of glycerol by *Streptococcus faecalis*. It is to be noted that, in all of these cases, the growth is abundant even though H_2 is not produced as a metabolic product. From these results it is evident that H_2 is found in growing cultures of *R. rubrum* only under certain specific conditions. Since NH_4Cl and yeast extract (or peptone) were routinely added to culture media for nonsulfur purple bacteria in the past, it is not surprising that the production of H_2 was not previously observed.

The tentative conclusion that both an amino acid and a dicarboxylic acid were required for H_2 evolution by *R. rubrum* (SI)[6] had been reached on the basis of the growth experiments described at the beginning of this section. However, in view of the ammonia inhibition it is apparent that the tests with ammonia as a nitrogen source nullified the conclusions drawn initially. Experiments with resting cells have shown that malic or fumaric acids alone can initiate the production of H_2 and that the phenomenon is completely inhibited by the addition of ammonia (Gest and Kamen, 1949; Gest, Kamen, and Bregoff, 1949). Succinic acid appears to be an exceptional substrate—in this particular case, the simultaneous presence of an amino acid seems to be necessary for H_2 production. In any event, this unusual combination of circumstances emphasizes the caution that must be exercised in attempting to deduce mechanisms from the results of growth experiments only.

It may be remarked in passing that a large number of substrates including fatty acids, sugars, purines, alcohols, and amino acids have been found to be ineffective in evoking photohydrogen production by resting cells (Gest, Kamen, and Bregoff, 1949).

Fixation of molecular nitrogen. An extremely interesting new aspect of the nitrogen metabolism of *R. rubrum* has been uncovered with the finding that this organism can incorporate molecular nitrogen into cell material when illuminated in a medium containing malate, biotin, and mineral salts (Kamen and Gest, 1949). This incorporation of N_2 is negligible if ammonia is added or if the organisms are incubated in the dark. These facts together with the observations that ammonia and molecular nitrogen inhibit photochemical H_2 formation by resting cells make it very probable that *R. rubrum* will be of great value for further investigation of the obscure relationship between hydrogenase activity and nitrogen fixation previously noted in other microorganisms (Wilson and Burris, 1947).

DISCUSSION

A more extensive investigation of the conditions required for observing H_2 formation in growing cultures is desirable but has been temporarily relinquished

[6] This strain of *R. rubrum* is unable to utilize nitrate as a nitrogen source and differs from the strain studied by Hutner (1944) in at least two respects—the latter can metabolize glucose and apparently cannot use ammonia.

in favor of resting cell experiments because of the difficulties already noted in interpreting the results of growth experiments. An investigation of the ubiquity of photochemical H_2 production in other species of nonsulfur purple bacteria and its possible occurrence in the sulfur purple bacteria will also be of decided interest from the viewpoint of comparative microbiology.

Previous work by Roelofsen (1935) with resting cell suspensions of a sulfur purple bacterium demonstrated a dark autofermentative evolution of H_2 that could not be augmented by the addition of various substrates. The relationship of Roelofsen's observations to those reported here is not clear. It is probable that H_2 can be produced by purple bacteria by several mechanisms, some of which are entirely independent of light. This opinion is further supported by the report that *Rhodobacillus palustris* and *Chromatium minutissimum* produce H_2 from formate, glucose, pyruvate, glycerol, and glycerophosphate in the dark (Nakamura, 1937, 1939). We have also observed dark production of H_2 from formate by *R. rubrum* (SI); if the organisms are grown in the presence of formate, they will decompose this compound under an atmosphere of N_2 without an appreciable adaptation period. The results of supplementary experiments with growing cultures and resting cell suspensions, however, make it very doubtful that formate (or pyruvate) is of significance as an intermediate in the photoproduction of H_2 (Gest, Kamen, and Bregoff, 1949).

Anaerobic production of H_2 from dicarboxylic acids by heterotrophic bacteria is well known (Barker, 1936, 1937; Tabachnick and Vaughn, 1948; Woods and Clifton, 1937). In these cases, other fermentation products such as volatile fatty acids are also formed. There is no evidence for products other than H_2, CO_2, and cells in the present instance. In fact, the equations frequently used for describing the metabolism, including growth, of purple bacteria are of the following type (Rabinowitch, 1945).

$$C_nH_{2n}(COOH)_2 + \frac{n-3}{2}CO_2 + \frac{n-1}{2}H_2O \rightarrow \frac{3n+1}{2}(CH_2O) - \sim(18n + 4)\text{kcal.}$$

Although this type of formulation may predict the order of magnitude of CO_2 evolution or consumption to be expected under certain conditions, it is obviously of questionable significance when H_2 is also formed as a metabolic product.

The inhibitory effect of ammonia on H_2 formation implies (1) that molecular hydrogen may be a normal intermediate that can function as a hydrogen donor for reductive amination as well as for CO_2 reduction, or (2) that molecular hydrogen may be in equilibrium with a reduced compound HX that can act as a hydrogen donor in metabolism. A study of amino acid synthesis under various conditions in these bacteria is contemplated in the near future. A mechanism for the production of H_2 by purple bacteria and its relation to a similar phenomenon occurring in green algae (Gaffron and Rubin, 1942) will be discussed in a forthcoming publication (Gest, Kamen, and Bregoff, 1949).

SUMMARY

The photosynthetic bacterium *Rhodospirillum rubrum* has been found to produce molecular hydrogen in addition to CO_2 during anaerobic growth in a synthetic medium containing glutamate, fumarate, biotin, and mineral salts. Fumaric acid is replaceable by malate or succinate. Low concentrations of yeast extract effect a marked stimulation of growth and gas production, presumably by furnishing unknown substances required for optimal development. The photoproduction of hydrogen is inhibited by ammonia and high concentrations of yeast extract and peptone. Observations on the inhibition of photohydrogen production have led also to the unexpected finding that *R. rubrum* is a nitrogen-fixing organism. The significance of these phenomena is discussed briefly, particularly in relation to the formation of hydrogen by other microorganisms.

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