JOURNAL OF BACTERIOLOGY, June 1980, p. 908-915
0021-9193/80/06-0908/08$02.00/0

Vol. 142, No. 3

# Physiology of Dark Fermentative Growth of *Rhodopseudomonas capsulata*

MICHAEL T. MADIGAN,†* JOHN C. COX, AND HOWARD GEST

*Photosynthetic Bacteria Group, Department of Biology, Indiana University, Bloomington, Indiana 47405*

The photosynthetic bacterium *Rhodopseudomonas capsulata* can grow under anaerobic conditions with light as the energy source or, alternatively, in darkness with D-fructose or certain other sugars as the sole source of carbon and energy. Growth in the latter mode requires an "accessory oxidant" such as trimethylamine-*N*-oxide, and the resulting cells contain the photosynthetic pigments characteristic of *R. capsulata* (associated with intracytoplasmic membranes) and substantial deposits of poly-β-hydroxybutyrate. In dark anaerobic batch cultures in fructose plus trimethylamine-*N*-oxide medium, trimethylamine formation parallels growth, and typical fermentation products accumulate, namely, $CO_2$ and formic, acetic, and lactic acids. These products are also found in dark anaerobic continuous cultures of *R. capsulata*; acetic acid and $CO_2$ predominate when fructose is limiting, whereas formic and lactic acids are observed at elevated concentrations when trimethylamine-*N*-oxide is the limiting nutrient. Evidence is presented to support the conclusions that ATP generation during anaerobic dark growth of *R. capsulata* on fructose plus trimethylamine-*N*-oxide occurs by substrate level phosphorylations associated with classical glycolysis and pyruvate dissimilation, and that the required accessory oxidant functions as an electron sink to permit the management of fermentative redox balance, rather than as a terminal electron acceptor necessary for electron transport-driven phosphorylation.

Purple photosynthetic bacteria (*Rhodospirillaceae*) are remarkably versatile in regard to their capacities for obtaining growth energy through alternative mechanisms. Indeed, every major known type of energy conversion has been observed in this assemblage of bacteria (17, 21, 24, 25, 27). Among these organisms, *Rhodopseudomonas capsulata* stands out as a particularly interesting individual species as it is capable of growing in a number of photosynthetic and dark modes (17). In 1977, Yen and Marrs (31) demonstrated that *R. capsulata* can grow anaerobically in darkness in synthetic media with glucose as the sole energy and carbon source, provided that dimethyl sulfoxide (DMSO) was added. They observed that during growth under these conditions, DMSO was reduced to dimethyl sulfide, but the nature of the energy conversion process supporting growth was not defined. We extended their studies on dark anaerobic growth of *R. capsulata* with fructose as the carbon and energy source and trimethylamine-*N*-oxide (TMAO) as the "accessory oxidant." Among other findings, we established that at least part of the system involved in TMAO reduction is inducible (16) and that the energy yield from the dark anaerobic catabolism of fructose is sufficient to support growth on $N_2$ as the sole source of nitrogen (18).

The present communication summarizes more detailed studies on the character of the energy conversion process(es) available to cells of *R. capsulata* growing on sugars plus TMAO anaerobically in darkness. Our results support the conclusions that ATP generation in such circumstances is fermentative (that is, mediated by substrate level phosphorylation associated with glycolysis and related reactions) and that TMAO functions as an electron sink so as to ensure redox balance (and not as an electron acceptor for membrane-associated anaerobic respiration coupled with phosphorylation).

## MATERIALS AND METHODS

**Bacterial strain.** *R. capsulata* strain B10, which was used throughout, conforms to the typical biotype of the species (29); it is capable of growing photosynthetically with various sugars or dicarboxylic acids of the citric acid cycle as carbon sources.

**Media.** For batch culture dark anaerobic growth experiments, the basal mineral salts plus vitamin medium CA (17) was modified so as to contain half the originally specified concentration of phosphate buffer (pH 6.8) and was supplemented with sugars and other compounds as indicated in table and figure legends. Concentrated stock solutions of TMAO (Aldrich

† Present address: Department of Microbiology, Southern Illinois University, Carbondale, IL 62901.

Chemical Co., Milwaukee, Wis.) and D-fructose were sterilized by filtration. For the continuous culture (dark anaerobic) experiments, the medium described by Aiking and Sojka (1) was modified by increasing the concentration of EDTA from 0.05 to 0.5 mM and replacing L-malate with fructose and TMAO (both sterilized separately by filtration) at the concentrations specified in Table 2 (initial pH, 7.0).

The RCVB medium (28) used for anaerobic photosynthetic growth contains 30 mM malate and 7.5 mM ammonium sulfate as the carbon and nitrogen sources, respectively.

**Growth conditions and sampling techniques.** Small-scale cultures were grown in completely filled screw-capped tubes (17 ml) or bottles (165 ml). For larger-scale anaerobic dark batch culture experiments in which TMAO was added as an accessory oxidant, bottles containing 850 ml of medium were inoculated with 50 ml of an exponential-phase "preculture" grown photosynthetically in RCVB medium supplemented with 20 to 30 mM TMAO. To facilitate intermittent anaerobic sampling, such bottles were sealed with a rubber stopper fitted with two stainless steel gas-sparging needles. After inoculation, oxygen-free $N_2$ was bubbled through the culture for 30 min at a rate of 200 ml/min (16). A sterile 20-ml glass syringe was then attached to the gassing inlet while the vent (short needle) was sealed with a rubber tube pinched off with a hose clamp. To remove a culture sample anaerobically, sterile $N_2$ was passed in through the vent needle, thus forcing bacterial suspension into the removable glass syringe.

For continuous culture, the apparatus devised by Aiking and Sojka (1) was used. This was installed in a dark incubator room and covered with a blackened box. During growth, the production of trimethylamine (TMA) from TMAO tends to increase the culture pH; accordingly, pH was maintained at 7.0 by means of a pH control unit which delivered 1 M HCl to the culture. The chemostat was maintained and the samples were taken as described in reference 1.

**Measurement of bacterial growth and cellular components.** Turbidimetric estimations of bacterial growth were routinely made with a Klett-Summerson photometer fitted with a no. 66 (red) filter. Measurements of protein, bacteriochlorophyll, and cell dry weight, as well as determinations of in vivo absorption spectra, were performed as previously described (17). Poly-$\beta$-hydroxybutyrate was measured by the method of Law and Slepecky (14).

**Chemical analyses.** Samples of bacterial culture were immediately centrifuged at 27,000 × *g* for 15 min, and the supernatant fluids were kept at 2°C until analysis. Fructose was determined with the anthrone procedure of Morris (19), and 2-keto-3-deoxy-6-phosphogluconate was detected by the method of Lanning and Cohen (13). TMA and lactate were estimated by the colorimetric methods of Dyer (7) and Barker and Summerson (4), respectively. Acetate was determined by gas chromatography on a Carbopack C/Carbowax 20 M column (Supelco, Inc., Bellefonte, Pa.) operating at 110°C with a flame ionization detector. Carbon dioxide was determined by gas chromatography on a Hewlett-Packard 5710A gas chromatograph with a Spherocarb 80/100 column and a thermal conductivity detector.

Formate was determined by the colorimetric assay of Barker and Somers (3), except that 2 ml, instead of 1.5 ml, of acidic butanol was used to extract the chromophore. Medium blanks were carried through the same procedure to allow corrections for the small background absorbance observed with fructose-containing media. Acetate does not interfere (3), and we have established that neither lactate nor pyruvate produces the yellow color characteristically obtained with formate.

**Preparation of cell-free extracts.** Cells were harvested in the late exponential phase, washed once with 50 mM potassium phosphate buffer (pH 7.0), and resuspended in 5 to 10 ml of the buffer. The suspensions were passed once through a French pressure cell operated at ca. 20,000 lb/in$^2$ and 2°C. Crude extracts were centrifuged at 27,000 × *g* (4°C) for 10 min to remove unbroken cells and debris, and the highly pigmented supernatant fluid was recentrifuged at 120,000 to 144,000 × *g* (4°C) for 90 min. The resulting clear supernatant fluid was used immediately for enzyme assays.

**Enzyme assays.** All assays were carried out at 25°C. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) activity was measured by the method of Gibbs (11), modified by the addition of $NAD^+$ in place of $NADP^+$. Hexokinase (glucokinase, EC 2.7.1.2), 1-phosphofructokinase (EC 2.7.1.56), fructose-bisphosphate aldolase (EC 4.1.2.13), 6-phosphogluconate dehydratase (EC 4.2.1.12), and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) were assayed by the methods used by Conrad and Schlegel (6). Substrate for the latter assay was a generous gift from Jack Preiss, Department of Biochemistry and Biophysics, University of California, Davis. Pyruvate reductase (EC 1.1.1.27) was assayed by the method of Gardner and Lascelles (10).

## RESULTS

**Substrate requirements for anaerobic dark growth.** With fructose or glucose as the sole source of carbon and energy for anaerobic dark growth, either TMAO or DMSO is also required (16, 31). The influence of initial fructose and TMAO concentrations on *R. capsulata* cell yield is shown in Fig. 1. With the TMAO concentration fixed at 30 mM, cell yield was proportional to the fructose concentration up to ca. 20 mM; a further increase in the sugar concentration did not alter the final yield. With the initial fructose concentration at 20 mM, the growth yield was proportional to the TMAO concentration up to about 40 mM; higher concentrations of the oxidant were inhibitory. Optimal growth rates were consistently observed with 30 mM TMAO, and this concentration was used for most experiments.

A survey of potentially fermentable carbohydrates and other compounds disclosed that growth in the presence of 30 mM TMAO was supported best by fructose. Other organic carbon sources that gave appreciable growth (but less than with fructose) were, in decreasing order of

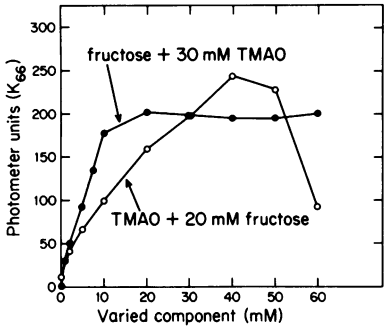


FIG. 1. *Anaerobic dark growth of R. capsulata, cell yields as a function of fructose and TMAO concentrations. Inoculated cultures, in completely filled screw-capped tubes, were incubated at 32°C for 30 h, and bacterial densities were then measured with a Klett-Summerson photometer equipped with a no. 66 filter; 200 $K_{66}$ units correspond to ca. 650 μg (dry weight) of cells per ml. The inoculum for this and the experiments shown in Fig. 2 and 3 were taken from cultures which had grown at least 12 to 15 generations under anaerobic dark conditions.*

efficacy, xylose, glucose, cellobiose, mannitol, pyruvate, and lactate. Slight growth was noted with sucrose, maltose, galactose, and ribose. It is particularly significant that no growth was observed with malate or succinate anaerobically in darkness (±TMAO); these organic acids, on the other hand, support excellent growth under respiratory (dark aerobic) and photosynthetic (light anaerobic) conditions (17, 27). *R. capsulata* can use $H_2$ to provide reducing power for photosynthetic growth on $CO_2$ and as the sole energy and electron source for aerobic autotrophic growth (17). Tests for the use of $H_2$ as an energy source for dark anaerobic growth with TMAO (on $CO_2$ or appropriate organic compounds), however, were negative.

**Specificity of the accessory oxidant requirement for anaerobic dark growth.** One implication of the foregoing results is that anaerobic dark growth on sugars is dependent on either ATP produced by substrate level phosphorylation in a fermentation process that requires TMAO or DMSO for redox balance or ATP generated by an electron transport chain that uses TMAO (or DMSO) as the final oxidant for oxidative phosphorylation. We reported earlier (16) that fumarate and nitrate, which can serve as terminal electron acceptors for anaerobic respirations catalyzed by various other bacteria (23), cannot substitute for TMAO (or DMSO). Additional tests with nitrite, sulfite, thiosulfate, and sulfate likewise gave negative results. Although the most effective oxidants known thus far are TMAO and DMSO, preliminary tests have shown that certain analogues of TMAO (including nicotinic acid-*N*-oxide, nicotinamide-*N*-oxide, and pyridine-*N*-oxide) also can function as accessory oxidants.

**Photosynthetic pigments and reserve materials.** Uffen and Wolfe (25) have reported that cells of several species of nonsulfur purple bacteria (*Rhodospirillaceae*) grown anaerobically in darkness in complex media show essentially the same pigment spectra as cells grown photosynthetically in the absence of oxygen. We have made similar observations with *R. capsulata* grown anaerobically in darkness in the synthetic fructose plus TMAO medium (16). Also in conformity with the results of Uffen and Wolfe with other species (25), we found (16) substantial quantities of poly-β-hydroxybutyrate in *R. capsulata* cells grown as noted. In the present investigation, analyses with the procedure of Law and Slepecky (14) showed that the storage polymer can accumulate to as much as 20% of the total dry weight.

**End products of anaerobic fructose catabolism in the presence of TMAO.** Figure 2 shows the kinetics of anaerobic dark growth of *R. capsulata* and organic acid formation in the 20 mM fructose plus 30 mM TMAO medium. TMA production from TMAO closely paralleled growth, and both ceased abruptly at ca. 15 h, the point of TMAO exhaustion (vertical dashed line). The maximal cell yield of ca. 200 photometer ($K_{66}$) units corresponds to 650 μg (dry weight) of cells per ml. It is important to note that fructose continued to disappear after growth had stopped; thus, after 15 h, fructose was metabolized by resting (nongrowing) cells.

Figure 2 (top) shows that during active growth, the culture pH rose due to the formation of the basic TMA. As growth proceeded, formate, acetate, and lactate began to accumulate with the kinetics indicated. Acetate was produced from the onset of growth, whereas formate production began in the mid log phase. Upon exhaustion of TMAO, the rates of production of these acids decreased sharply; in contrast, lactate formation commenced and continued for many hours with a stoichiometry that approximated a simple homolactic fermentation (that is, ca. 2 mol of lactate per mol of fructose used; see Table 1). The decrease in pH that began at 15 h was evidently due mainly to lactic acid formation.

In addition to the products already discussed, $CO_2$ was formed in substantial amounts during anaerobic dark growth on fructose plus TMAO, and trace amounts of 2-keto-3-deoxy-6-phosphogluconate were also detected. The data in Table 1 show the relative amounts of all the major end products formed in an experiment in which sam-

ples were taken for analysis at 15 h (TMAO exhausted) and at 30 h. Before the exhaustion of TMAO in such experiments, the calculated molar growth yield was quite high, namely, of the order of 50 to 60 g (dry weight) of cells per mol of fructose consumed.

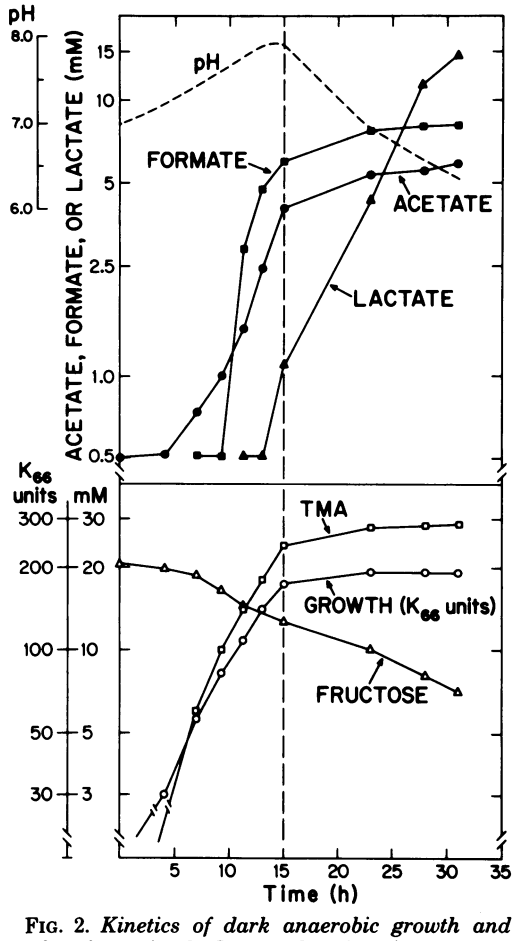


FIG. 2. *Kinetics of dark anaerobic growth and product formation by R. capsulata in a fructose plus TMAO medium. Concentrations of products are given as mM quantities; culture turbidity (growth) is expressed as $K_{66}$ units.*

**Competition between pyruvate and TMAO for reducing equivalents.** The kinetics of TMA and lactate formation suggest that reducing equivalents generated during anaerobic fructose catabolism can be used for the reduction of pyruvate to lactate but are preferentially diverted to TMAO when the oxidant is present. This was tested by examining the effects of providing additional TMAO (20 mM) to a batch culture in the stationary phase after exhaustion of the TMAO added initially.

In Fig. 3 it is seen that the supplemental addition of TMAO to a culture in the stationary phase at 41 h led to the resumption of growth and TMA formation; lactate production, however, was immediately inhibited. After the exhaustion of the TMAO supplement, lactate excretion resumed, as in the first part of the experiment. The course of pH changes shown at the top of Fig. 3 clearly reflects whether TMA or lactic acid is being actively produced. These results indicate that there is a competition between pyruvate and TMAO for reducing equivalents (NADH) generated from fructose catabolism. It is still not known why the homolactic fermentation of fructose that occurs after the disappearance of TMAO cannot support additional growth under the experimental conditions described.

Further evidence for the competition between TMAO and pyruvate for electrons from NADH was obtained from continuous culture experiments in which either TMAO or fructose was made the limiting nutrient for dark anaerobic growth. Preliminary trials showed that with either limitation cell density remained constant (at ca. 300 μg [dry weight] of cells per ml) with increasing dilution rate until about 0.075 $h^{-1}$; with a further increase in the dilution rate, cell density decreased rapidly, and washout occurred at a dilution rate of 0.10 to 0.11. For the experiments shown in Table 2, a dilution rate of 0.05 $h^{-1}$ was used, corresponding to a doubling time of 14 h. With TMAO limiting, lactate and formate predominated as fermentation products;

TABLE 1. *Products of batch culture growth of R. capsulata on fructose plus TMAO anaerobically in darkness*[a]

| Time (h) | Substrate | | Product | | | | | Cell dry weight (μg/ml) | Cell carbon[c] (μg/ml) | Total fructose carbon consumed (μg/ml) | Calculated carbon recovery (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fructose | TMAO | $CO_2$ | Formate | Acetate | Lactate | KDPG[b] (μM) | | | | |
| 0 | 20 | 30 | 0 | 0 | 0 | 0 | 0 | 10 | 5.5 | 0 | |
| 15 | 11 | 0 | 12 | 6.5 | 4.5 | 1.0 | 0.15 | 636 | 350 | 648 | 109 |
| 30 | 6 | 0 | 12 | 7.5 | 5.5 | 12.5 | 0 | 575 | 316 | 1,008 | 111 |

[a] Unless otherwise noted, values are mM quantities.
[b] KDPG, 2-keto-3-deoxy-6-phosphogluconate.
[c] Calculated assuming carbon to be 55% of the cell dry weight (compare reference 26).

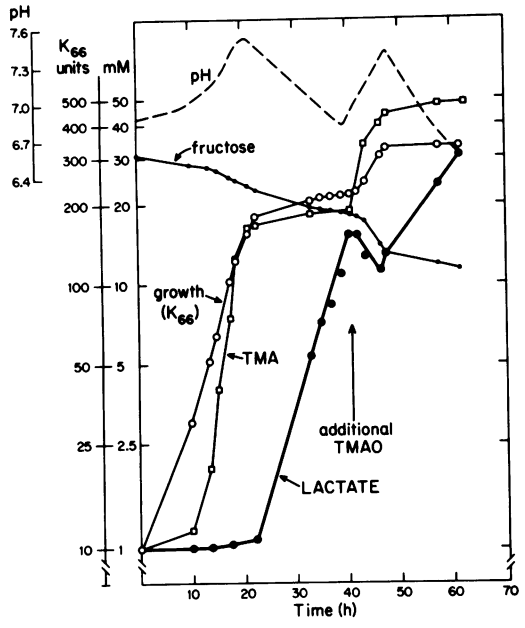


FIG. 3. *Dark fermentative metabolism of fructose by R. capsulata; effects of additional TMAO on growth and lactate formation. The results shown are for an experiment similar to that shown in Fig. 2; at 41 h (vertical arrow), additional TMAO (30 mM) was added anaerobically.*

TABLE 2. *Metabolic products from continuous cultures of R. capsulata grown anaerobically in darkness with fructose or TMAO limiting*

| Limiting nutrient | Product (μg of carbon per ml of culture) | | | | |
|---|---|---|---|---|---|
| | Acetate | $CO_2$ | Formate | Lactate | Cells[a] |
| Fructose[b] | 125 | 80 | 26 | ~0 | 77 |
| TMAO[c] | 38 | 31 | 72 | 159 | 94 |

[a] Calculated assuming carbon to be 55% of the cell dry weight (26).

[b] Medium reservoir contained 4 mM fructose and 30 mM TMAO; the TMAO concentration at steady state was 13 mM.

[c] Medium reservoir contained 8 mM TMAO and 20 mM fructose; the fructose concentration at steady state was 15.2 mM.

acetate and $CO_2$, which result from the electron acceptor-dependent metabolism of pyruvate, were present, but in considerably lower amounts. Conversely, with the electron acceptor (TMAO) in excess and fructose limiting, $CO_2$ and acetate predominated, and no lactate was detectable. The availability of excess TMAO as an electron sink obviously favored the oxidative conversion of the fermentation intermediate pyruvate to $CO_2$ and acetate. The latter process ordinarily involves the production of acetyl coenzyme A as an intermediate and, consequently, increases the bioenergetic potential of the fermentation.

**Enzymes of fructose catabolism in extracts of fermentatively grown cells.** Conrad and Schlegel (6) have assayed levels of pertinent enzymes in extracts of cells of *R. capsulata* grown photosynthetically with fructose as the carbon source and concluded that under the conditions noted, fructose is metabolized to the $C_3$ stage via the Embden-Meyerhof-Parnas pathway. Table 3 lists specific activities of certain enzymes of sugar metabolism in extracts of cells grown anaerobically in darkness on fructose plus TMAO, that is, under conditions in which fructose is the sole source of energy as well as carbon. The high specific activities of 1-phosphofructokinase and fructose-bisphosphate aldolase and the absence (not indicated in the table) of fructokinase as well as 6-phosphofructokinase are consistent with the operation of the pathway

fructose → fructose-1-phosphate
→ fructose-bisphosphate
→ dihydroxyacetone phosphate
+ glyceraldehyde-3-phosphate.

As observed by Conrad and Schlegel (6) with photosynthetically grown cells, we also detected enzymes of the Entner-Doudoroff pathway in extracts of cells grown fermentatively on fructose; these, however, were present at considerably lower specific activities (Table 3).

As expected, the specific activity of triosephosphate dehydrogenase was also very high (Table 3). This enzyme was found to be specific for $NAD^+$, and as assayed in crude extracts, its

TABLE 3. *Specific activities of certain enzymes of sugar metabolism in extracts of R. capsulata cells grown fermentatively on fructose plus TMAO*[a]

| Enzyme | Sp act (nmol/min per mg of protein) |
|---|---|
| Embden-Meyerhof-Parnas pathway | |
| 1-phosphofructokinase | 158 |
| fructose-bisphosphate aldolase | 252 |
| hexokinase | 37 |
| Entner-Doudoroff pathway | |
| glucose-6-phosphate dehydrogenase | 25 |
| 6-phosphogluconate dehydratase | 48 |
| 2-keto-3-deoxy-6-phosphogluconate aldolase | 73 |
| glyceraldehyde-3-phosphate dehydrogenase | 410 |
| pyruvate reductase | 75 |

[a] The values given represent the composite from two separate experiments in which cells were harvested ca. 12 h after inoculation.

activity was strictly arsenate dependent. Thus, the triosephosphate dehydrogenase of fermentatively grown *R. capsulata* is of the classical type and not an unusual NADP-linked enzyme of the type observed in certain kinds of lactic acid bacteria (5). A soluble NADH-dependent pyruvate reductase activity was readily demonstrable in extracts of fermentatively grown cells (Table 3); the enzyme in such extracts showed a $K_m$ of ca. 170 μM and showed no activity in tests for lactate oxidation. The foregoing observations, taken together with the results relating to fermentation end products, support the conclusion that cells of *R. capsulata* growing anaerobically in darkness on fructose plus TMAO use the classical Embden-Meyerhof-Parnas glycolytic pathway as a major energy conservation mechanism.

## DISCUSSION

As TMAO functions as an electron acceptor during anaerobic dark growth on sugars, the possibility that *R. capsulata* can produce ATP by anaerobic respiration with TMAO as the terminal oxidant must be considered. Several lines of evidence cogently argue against such interpretation and are briefly summarized here. (i) Only potentially fermentable substrates serve as energy sources for TMAO-dependent growth. Dicarboxylic organic acids associated with the aerobic citric acid cycle can be used as carbon and energy sources for aerobic dark growth of *R. capsulata* or as carbon sources for anaerobic photosynthetic development (17), but do not support anaerobic dark growth in the presence (or absence) of TMAO. (ii) Anaerobic respiration implies extensive degradation of organic energy sources, as in aerobic respiration; inorganic oxidants such as nitrate or elemental sulfur presumably only provide an electron sink alternative to molecular oxygen. Indeed, anaerobic bacteria such as *Desulfuromonas acetoxidans* use elemental sulfur as a terminal electron acceptor for the total oxidation of acetate to $CO_2$ (20). In contrast, anaerobic dark growth of *R. capsulata* on fructose plus TMAO is characterized by the formation of classical fermentation end products in substantial amounts, as in numerous fermentations catalyzed by heterotrophic anaerobes or facultative anaerobes (23). In addition, energy conversion in anaerobic respiration is now understood to represent oxidative phosphorylation driven by electron flow. (iii) A separate investigation to be detailed elsewhere (J. C. Cox, M. T. Madigan, J. L. Favinger, and H. Gest, Arch. Biochem. Biophys., in press) has established that the electron transport system responsible for the oxidation of NADH by TMAO in *R. capsulata* is localized in the cytoplasmic (soluble) fraction of the cell. Although it is known that certain bacteria contain a membrane-associated electron transport system that can use TMAO as an electron acceptor (22, 30), the inducible TMAO reduction system of *R. capsulata* is of a fundamentally different character.

The foregoing considerations indicate that the function of TMAO (and DMSO) in the dark anaerobic growth of *R. capsulata* on sugars is to provide an electron sink only for the purpose of achieving redox balance. *R. capsulata* is unable to produce products of a very reduced nature (such as $H_2$, ethanol, or long-chain fatty acids) from the anaerobic dark dissimilation of sugars under the nutritional conditions employed, and although resting cells can catalyze the homolactic fermentation of hexose, growing cells appear unable to manage redox balance in this fashion. Thus, TMAO (or DMSO) apparently functions simply as an electron acceptor for the reoxidation of NADH generated by the triosephosphate dehydrogenase reaction of the Embden-Meyerhof-Parnas pathway, providing the $NAD^+$ necessary for continued fermentation, that is, for the production of ATP by substrate level phosphorylation. Our observations also indicate that TMAO can serve as an electron acceptor for the oxidative conversion of pyruvate to acetyl coenzyme A plus $CO_2$, thereby making available additional ATP from the sequence

acetyl coenzyme A

$$\rightarrow \text{acetyl phosphate} \xrightarrow{+ADP} \text{acetate} + \text{ATP}$$

Nonoxidative cleavage of pyruvate to acetyl coenzyme A plus formate is bioenergetically equivalent, and Gorrell and Uffen (12) consider this to be a predominant energy source for anaerobic dark growth of *Rhodospirillum rubrum*. The extensive deposition of poly-β-hydroxybutyrate granules in cells of fermentatively grown *Rhodospirillaceae* indicates an abundant generation of acetyl coenzyme A in these circumstances.

A simplified representation of our interpretation of the fermentative metabolism of *R. capsulata* is shown in Fig. 4. Redox balance in typical fermentations is achieved through the reduction of intermediates (such as pyruvate) generated from the catabolism of the original substrate. As depicted in Fig. 4, fermentative reactions in *R. capsulata* involve the reduction of an externally supplied electron acceptor such as TMAO or DMSO and thus allow a greater conversion of pyruvate to acetate, with subsequent production of additional ATP. Although it might seem at first glance that non-phosphorylative oxidation of NADH by an exogenous oxidant is a very unusual and possibly anoma-

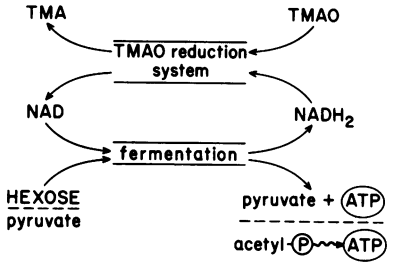


FIG. 4. *Schematic representation of the fermentative metabolism of R. capsulata.*

lous mechanism for achieving redox balance in fermentations, processes of this kind may be much more common than is usually supposed. Thus, exogenous acetate is used as an accessory electron acceptor in fermentations by certain clostridia and is reduced to butyrate, which accumulates in the medium (2). Other clostridia can achieve redox balance in sugar fermentations by reducing $CO_2$ to acetate (15). The concept of an accessory oxidant-dependent reoxidation of NADH has also been invoked to explain the action of inorganic nitrate in promoting fermentation by *Clostridium welchii* (8). In all of these instances, the function of the accessory oxidant, apparently, is to provide an electron sink for the reoxidation of NADH, thereby providing the $NAD^+$ required for the oxidative energy-yielding reactions of fermentation.

DMSO and several other sulfoxides (methionine and biotin sulfoxides) occur naturally in certain environments (32, 33), whereas TMAO is produced by various marine animals and is thought to be one of the most common organic compounds in the oceans (9). Other substances containing the *N*-oxide moiety (such as nicotine- and nicotinamide-*N*-oxides) are also found in terrestrial and freshwater habitats (9). Such compounds, and possibly others yet to be discovered, may play significant ecological roles by facilitating dark fermentative growth of photosynthetic bacteria. The capacity for metabolism and growth anaerobically in darkness presumably would provide some competitive advantage for photosynthetic bacteria in oxygen-free habitats that are light-limited or intermittently dark.

## ACKNOWLEDGMENTS

This research was supported by National Science Foundation grant PCM 76-08886. H.G. is the recipient of a fellowship from the John Simon Guggenheim Memorial Foundation. J.C. acknowledges the Fulbright-Hays Commission and the Wellcome Foundation for travel support.

We thank Jeff Favinger and Michael Scott for skilled technical assistance, Thomas Beatty for helpful discussions, and William Qualls for help and advice in the measurement of $CO_2$.

## LITERATURE CITED

1. **Aiking, H., and G. Sojka.** 1979. Response of *Rhodopseudomonas capsulata* to illumination and growth rate in a light-limited continuous culture. J. Bacteriol. **139:** 530–536.
2. **Barker, H. A.** 1956. Bacterial fermentations. John Wiley & Sons, Inc., New York.
3. **Barker, S. A., and P. J. Somers.** 1966. A spectrophotometric method for the determination of formic acid in the periodate oxidation of carbohydrates. Carbohy. Res. **3:**220–224.
4. **Barker, S. B., and W. H. Summerson.** 1941. The colorimetric determination of lactic acid in biological materials. J. Biol. Chem. **138:**535–554.
5. **Chow, V. L., and C. L. Wittenberger.** 1979. Separation and properties of $NAD^+$ and $NADP^+$ dependent glyceraldehyde-3-phosphate dehydrogenases from *Streptococcus mutans*. J. Biol. Chem. **254:**1134–1142.
6. **Conrad, R., and H. G. Schlegel.** 1977. Different degradation pathways for glucose and fructose in *Rhodopseudomonas capsulata*. Arch. Microbiol. **112:**39–48.
7. **Dyer, W. J.** 1959. Report on trimethylamine in fish. J. Assoc. Off. Agric. Chem. **42:**292–294.
8. **Egami, F., K. Ohmachi, K. Iida, and S. Taniguchi.** 1957. Nitrate reducing systems in cotyledons and seedlings of bean seed embryos *Vigna sesquipedalis* during the germinating stage. Biokhimiya. **22:**122–134.
9. **Forster, R. P.** 1972. Excretion of foreign nitrogenous compounds, p. 235–276. *In*: J. W. Campbell and L. Goldstein (ed.), Nitrogen metabolism and the environment. Academic Press, Inc., London.
10. **Gardner, J. F., and J. Lascelles.** 1962. Requirement for acetate of a streptomycin-resistant strain of *Staphylococcus aureus*. J. Gen. Microbiol. **29:**157–164.
11. **Gibbs, M.** 1955. TPN triosephosphate dehydrogenase from plant tissue. Methods Enzymol. **1:**411–415.
12. **Gorrell, T. E., and R. L. Uffen.** 1977. Fermentative metabolism of pyruvate by *Rhodospirillum rubrum* after anaerobic growth in darkness. J. Bacteriol. **131:** 533–543.
13. **Lanning, M. C., and S. S. Cohen.** 1951. The detection and estimation of 2-ketohexonic acids. J. Biol. Chem. **189:**109–114.
14. **Law, J. H., and R. A. Slepecky.** 1961. Assay of poly-$\beta$-hydroxybutyric acid. J. Bacteriol. **82:**33–36.
15. **Ljungdahl, L. G., and H. G. Wood.** 1969. Total synthesis of acetate from $CO_2$ by heterotrophic bacteria. Ann. Rev. Microbiol. **23:**515–538.
16. **Madigan, M. T., and H. Gest.** 1978. Growth of a photosynthetic bacterium anaerobically in darkness, supported by "oxidant-dependent" sugar fermentation. Arch. Microbiol. **117:**119–122.
17. **Madigan, M. T., and H. Gest.** 1979. Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with $H_2$ as the energy source. J. Bacteriol. **137:**524–530.
18. **Madigan, M. T., J. D. Wall, and H. Gest.** 1979. Dark anaerobic dinitrogen fixation by a photosynthetic microorganism. Science **204:**1429–1430.
19. **Morris, D. L.** 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science **107:**254–255.
20. **Pfennig, N., and H. Biebl.** 1976. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing acetate-oxidizing bacterium. Arch. Microbiol. **110:**3–12.
21. **Satoh, T., Y. Hashino, and A. Kitamura.** 1976. *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*, a denitrifying strain as a subspecies of *Rhodopseudomonas sphaeroides*. Arch. Microbiol. **108:**265–269.
22. **Strøm, A. R., J. A. Olafsen, and H. Larsen.** 1979. Trimethylamine oxide: a terminal electron acceptor in anaerobic respiration of bacteria. J. Gen. Microbiol. **112:**315–320.
23. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacte-

ria. Bacteriol. Rev. **41**:100–180.

24. **Uffen, R. L.** 1976. Anaerobic growth of a *Rhodopseudomonas* species in the dark with carbon monoxide as sole carbon and energy source. Proc. Natl. Acad. Sci. U.S.A. **73**:3298–3302.
25. **Uffen, R. L., and R. S. Wolfe.** 1970. Anaerobic growth of purple nonsulfur bacteria under dark conditions. J. Bacteriol. **104**:462–472.
26. **van Niel, C. B.** 1936. On the metabolism of the *Thiorhodaceae*. Arch. Mikrobiol. **7**:323–358.
27. **van Niel, C. B.** 1944. The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. Bacteriol. Rev. **8**:1–118.
28. **Wall, J. D., B. C. Johansson, and H. Gest.** 1977. A pleiotropic mutant of *Rhodopseudomonas capsulata* defective in nitrogen metabolism. Arch. Microbiol. **115**: 259–263.
29. **Weaver, P. F., J. D. Wall, and H. Gest.** 1975. Characterization of *Rhodopseudomonas capsulata*. Arch. Microbiol. **105**:207–216.
30. **Yamamoto, I., and M. Ishimoto.** 1977. Anaerobic growth of *Escherichia coli* on formate by reduction of nitrate, fumarate, and trimethylamine-N-oxide. Z. Allg. Mikrobiol. **17**:235–242.
31. **Yen, H.-C., and B. Marrs.** 1977. Growth of *Rhodopseudomonas capsulata* under anaerobic dark conditions with dimethyl sulfoxide. Arch. Biochem. Biophys. **181**: 411–418.
32. **Zinder, S. H., and T. D. Brock.** 1978. Dimethyl sulfoxide reduction by microorganisms. J. Gen. Microbiol. **105**: 335–342.
33. **Zinder, S. H., and T. D. Brock.** 1978. Dimethyl sulfoxide as an electron acceptor for anaerobic growth. Arch. Microbiol. **116**:35–40.