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Chemoautotrophic Growth of *Rhodopseudomonas* Species with Hydrogen and Chemotrophic Utilization of Methanol and Formate

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Abstract. A chemolithoautotrophic type of metabolism, which was hitherto unknown for purple nonsulfur bacteria, was demonstrated by growth experiments using *Rhodopseudomonas capsulata* Kb1 and *Rhodopseudomonas acidophila* 10050. These strains were able to grow in a mineral medium in the dark at the expense of H_2, O_2, and CO_2. A minimum doubling time of 9 h was obtained for *R. capsulata* under an atmosphere containing less than 15% oxygen; higher oxygen concentrations suppressed autotrophic but not chemoorganotrophic growth. Oxygen sensitivity of chemoautotrophically growing cells of *R. acidophila* was even more pronounced, whereas cells growing chemotrophically on methanol almost tolerated the oxygen concentration of air. Highest oxygen sensitivity of growth of *R. acidophila* was observed with formate as substrate. The growth yield of cultures grown semiaerobically in the dark on methanol was 0.23 g dry cell material per g methanol consumed.

Key words: *Rhodopseudomonas capsulata* – *Rhodopseudomonas acidophila* – Chemolithoautotrophic growth – Methylotrophic growth – Oxygen sensitivity of growth – Hydrogenase activity – Methanol – Formate.

The purple nonsulfur bacteria exhibit a variety of nutritional types; they grow best under photoorganotrophic conditions. Most species also grow chemoorganotrophically in the dark. Several species have been shown to grow photoautotrophically with inorganic electron donors, particularly with H_2 (Ormerod and Gest, 1962; Klemme, 1968; Pfennig, 1969). In this connection it is of interest that *Rhodopseudomonas acidophila* and other purple nonsulfur bacteria are capable of anaerobic phototrophic growth in a methanol/bicarbonate medium (Quayle and Pfennig, 1975). From experimental data Sahm et al. (1976) concluded that *R. acidophila* oxidizes methanol to CO_2 and uses the reducing equivalents for CO_2-fixation via the ribulose bisphosphate cycle. Although *R. acidophila* can utilize a large number of simple organic compounds for aerobic growth in the dark, no aerobic growth could be observed with methanol in the dark. To explain this failure Sahm et al. (1976) discussed two possibilities: Firstly, the increased K_m-value which the methanol-dehydrogenase exhibited in the presence of air and, secondly, the repression of ribulose bisphosphate carboxylase under aerobic conditions (Lascelles, 1960). In both cases a high oxygen concentration would be the reason for growth inhibition. Therefore, growth should be possible under reduced oxygen partial pressure. Semiaerobic methylotrophic growth has indeed been demonstrated. Further growth studies, in which methanol was substituted by H_2 as electron donor led to the discovery of chemoautotrophic growth of *Rhodopseudomonas capsulata* and *R. acidophila* under micro- to semiaerobic conditions (Pfennig and Siefert, 1977; Siefert, 1978).

In the present paper the details of the experimental conditions for chemoautotrophic growth of *R. capsulata* are described. In addition micro- to semiaerobic growth of *R. acidophila* 10050 on hydrogen, methanol or formate as energy source is reported and compared with respect to oxygen tolerance.

Material and Methods

Organisms and Media. Rhodopseudomonas capsulata Kb1 (DSM 155) and *R. acidophila* 10050 (DSM 145) of the departmental collection were used. The minimal medium for *R. capsulata* contained per liter of distilled water: 0.5 g KH_2PO_4; 0.4 g $MgSO_4 \cdot 7\,H_2O$; 0.4 g NaCl; 1 g NH_4Cl; 0.05 g $CaCl_2 \cdot 2\,H_2O$; 1 ml trace element solution SL8

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(Biebl and Pfennig, 1978) and 10 ml vitamin solution (Pfennig, 1965). The pH was adjusted to 6.9 with a solution of 1 M Na_2CO_3. During pH adjustment the medium was permanently gassed with a mixture of 5% CO_2 in N_2. The medium was sterilized by filtration (Seitz-filter EKS). For *R. acidophila* the same medium was used, except that vitamins were omitted and the pH was adjusted to 6.0 for autotrophic growth. With methanol and formate as substrates the pH was 7.1 (Quayle and Pfennig, 1975).

Test for Oxygen Sensitivity. This was carried out in deep agar shake cultures as described by de Vries and Stouthamer (1969). The tubes were incubated in anaerobic jars with different mixtures of H_2, CO_2, and air as specified.

Growth Experiments. Growth experiments were run in a 10 l Biostat S fermenter (Braun, Germany) which was gassed with a mixture of H_2, O_2, CO_2 and N_2 and stirred at a rate of 400 rev/min. The temperature was kept constant at 30°C, and pH was controlled using an autoclavable electrode (Ingold). The appropriate mixture of the four gas components H_2, O_2, N_2 and CO_2 was obtained by means of gas mixing pumps (Wösthoff, Bochum) running at different pump rates. Constant concentrations of 80% H_2 and 5% CO_2 were maintained, whereas O_2 and N_2 concentrations were varied. At O_2 concentrations higher than 15% the H_2 flow rate was reduced correspondingly so that a constant overall flow rate of 1 l gas · min^{-1} was maintained. The fermenter was inoculated with 500 ml of a culture grown photoorganotrophically with 0.1% succinate as carbon-source.

Growth experiments with methanol or formate as substrate were run in a 2-l fermenter with oxygen control device. Since the growth yield on formate is low and growth is accompanied by a rise in pH, 10% formic acid was used to combine substrate addition and pH adjustment.

Growth Experiments with Controlled Oxygen Concentration. A 2-l fermenter equipped with a temperature and a pH control device was used. Throughout the growth period of a batch culture a constant oxygen tension was maintained by the following arrangements. The gas outlet of the fermenter was connected with the gas inlet via gas analysers and a pump using gas-tight Viton tube (Isoversinic) to obtain a closed gas circulation. By means of a transformer regulated membrane pump (Reciprotor 506 R, Edwards, Germany) the effluent gas was pumped through the system at a rate of 35 l/h. Dissolved oxygen concentration in the culture medium was measured by an autoclavable polarographic electrode (Ingold) and gaseous oxygen of the gas circulation was measured by a paramagnetic oxygen analyser (Servomex). Decrease of oxygen concentration caused by the respiration of the culture was compensated by controlled introduction of pure oxygen into the gas circulation via a magnetic valve. The valve opened automatically when the actual oxygen concentration was below the set point and closed when oxygen exceeded the set point. Thus, as desired, either dissolved oxygen tension or, alternatively, oxygen partial pressure of the gas phase could be maintained constant. The concentration of CO_2 was permanently measured by a CO_2-analyser (Uras, Hartmann and Braun) and maintained constant by passing the gas of the circulation through a solution of 10% KOH when the CO_2 content exceeded 5%.

Gaschromatographic Determination of Methanol. The sample of the culture was centrifuged and the methanol concentration of the supernatant was determined using a Perkin Elmer, F 22, gaschromatograph equipped with a flame ionisation detector. A volume of 5 µl was directly injected on a glass column (on-column injection) of 2 m length and 4 mm diameter packed with Porapak Q–S (100–120 mesh). At an oven temperature of 160°C and a flow rate of the carrier gas (N_2) of 30 ml/min the retention time of methanol was 85 s. The area of the methanol peak was proportional to the methanol concentration.

Respiration Rate of Autotrophically Grown Cells. Oxygen consumption of whole cells at 30°C was measured using a Clark type electrode connected to a Yellow Springs Instrument oxygen analyser. The cell suspension was obtained at an optical density of 0.65 (650 nm, 1 cm cuvette) from a culture growing autotrophically. The suspension was bubbled vigorously for 10 min with 80% H_2 and 20% O_2 prior to measurement. The electrode was calibrated with air saturated distilled water, which was assumed to contain 0.24 mmol $O_2 \cdot l^{-1}$ at 30°C.

Hydrogenase. This enzyme was determined in whole cells as described by Siefert and Pfennig (1978).

Bacteriochlorophyll. The specific bacteriochlorophyll content of whole cells was determined according to Siefert et al. (1978).

Calculation of the CO_2-fixation Rate of Growing Cells. The equation $\frac{ds}{dt \cdot x} = \frac{\mu}{y}$ (Pirt, 1975) was used to estimate the rate of CO_2-fixation. The specific growth rate (μ) was experimentally determined. A yield (y) of 23.8 g dry cell material per mol of CO_2 fixed was calculated assuming a carbon content of cell material of 50%.

Results

Chemolithoautotrophic Growth of Rhodopseudomonas acidophila in Deep Agar Shake Culture

Simple growth tests using the deep agar shake culture method (de Vries and Stouthamer, 1969; Pfennig, 1970) displayed after few days incubation the capacity of *Rhodopseudomonas acidophila* 10050 to grow in the dark on a mineral medium under an atmosphere of 50% H_2, 5% CO_2, and 45% air. The bacteria developed in a layer located 2–3 mm below the agar surface. In controls where H_2 was substituted by N_2, comparatively poor growth was observed even after prolonged incubation. With 30% H_2, 5% CO_2 and 65% air the distance of the growth layer from the surface was larger (4 mm) whereas cells provided with succinate as energy and carbon source grew close to the agar surface under air. Evidently, the chemoautotrophic growth is inhibited by the oxygen concentrations in equilibrium with 50%–100% air.

Chemolithoautotrophic Growth of Rhodopseudomonas capsulata in Liquid Culture

For growth experiments in liquid culture *Rhodopseudomonas capsulata* Kb1 was used, a strain which grows rapidly under photoautotrophic conditions (Klemme, 1968). On account of the observed microaerophily of *R. acidophila* 10050 under chemoautotrophic growth conditions, the batch culture experiment (Fig. 1) was started at low oxygen concentration. To adapt the oxygen supply to the increasing requirement of the growing culture, the oxygen concentration of the gas mixture was raised stepwise. A minimum doubling time of 9 h was obtained. The specific bacteriochlorophyll content of the cells was inversely related to the actual oxygen concentration (Fig. 1) similar as established for chemoorganotrophic growth

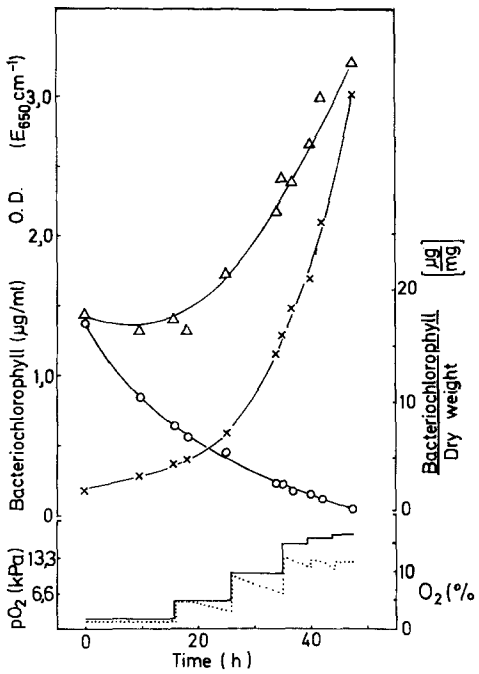

Fig. 1. Chemolithoautotrophic growth of *Rhodopseudomonas capsulata* Kb1 in the dark at the expense of H_2, O_2, and CO_2. The 10-l fermenter vessel was inoculated with 1 l of a culture grown anaerobically in the light on succinate. The minimum doubling time of *R. capsulata* under autotrophic conditions was 9 h. Optical density (×——×). Bacteriochlorophyll in µg per ml culture (△——△). Bacteriochlorophyll per bacterial dry weight (○——○). Oxygen concentration in the gas phase (solid line). Dissolved oxygen tension measured with an oxygen electrode in the culture medium (dotted line)

(Drews et al., 1969). Chemoautotrophic growth was not inhibited by oxygen concentrations which gradually repressed the synthesis of bacteriochlorophyll. From Fig. 2 it can be seen that chemoautotrophic growth was still possible at oxygen concentrations which did not effect a further decrease of the specific bacteriochlorophyll content (1 µg bacteriochlorophyll · mg^{-1} dry weight). Above 15% oxygen, however, chemoautotrophic growth was completely inhibited. Growth inhibition could be reversed when the oxygen concentration was lowered.

The oxygen concentration of air did not only affect the specific growth rate of chemoautotrophically growing cells but also their specific hydrogenase activity. Figure 3 shows that specific growth rate and hydrogenase activity changed in a coordinate manner after a sudden increase of the oxygen concentration. Hydrogenase was also suppressed in cells grown chemoorganotrophically on succinate at vigorous aeration. In contrast, cells grown photoorganotrophically in the absence of oxygen contained a basic level of specific hydrogenase activity of 0.6 µl · min^{-1} · mg^{-1} dry weight. We conclude that an oxygen sensitivity of the hydrogenase system may be the reason for the microaerophily of autotrophic growth, although we cannot exclude an additional effect of oxygen on the ribulose bisphosphate carboxylase activity (Lascelles, 1960; Codd and Turnbull, 1975). The efficiency of chemoautotrophic energy metabolism can be expressed by the ratio (n) between the rates of O_2 and CO_2 consumption (Aragno and Schlegel, 1978; Bongers, 1970). The maximum respiration rate of *R. capsulata* cells was determined polarographically to be 153 nmol O_2 · min^{-1} · mg^{-1} dry weight in the presence of 75% H_2 and 20% O_2 and 5% CO_2. A CO_2 fixation rate of 50 nmol CO_2 · min^{-1} · mg^{-1} dry weight at the maximum growth rate of 0.072 h^{-1} has been calculated. Thus a ratio $n = 3.0$ is obtained which is in the range of the values for the well known hydrogen bacteria (Bongers, 1970).

A stimulation of the Knallgas reaction by the addition of CO_2 ("Bartha-effect", Bartha, 1962) could not be observed. In the presence of 5% CO_2 the respiration rate was even reduced from 227 to 153 nmol O_2 · min^{-1} · mg^{-1} dry weight suggesting that O_2 and CO_2 compete for reducing equivalents from H_2.

Chemotrophic Growth of Rhodopseudomonas acidophila with CH_3OH, HCOOH and CO_2

Sahm et al. (1976) and Bamforth and Quayle (1978) did not obtain growth of *R. acidophila* aerobically with methanol as energy and carbon source. A similar oxygen sensitivity as observed for chemoautotrophic growth was suspected for chemotrophic growth on methanol. Three batch cultures, however, grown under controlled dissolved oxygen tensions of 50, 100, and, as shown in Fig. 4, at 120 Torr (= 6.6, 13.3, and 16.0 kPa respectively) were determined to have a specific growth rate of 0.02 h^{-1} irrespective of the oxygen tension. Even at an oxygen tension of 150 Torr (= 20.0 kPa) growth proceeded for four cell mass doublings until it became inhibited by oxygen. The growth inhibition could be reversed by decrease of oxygen concentration. All growth experiments were carried out in the presence of bicarbonate in equilibrium with 5% gaseous CO_2 at pH 7. In preliminary growth experiments without CO_2/bicarbonate aerobic growth was poor, apparently there is a definite requirement for exogenous CO_2 to obtain optimum growth. Bicarbonate was reported to stimulate methylotrophic growth of *Micrococcus denitrificans* (Cox and Quayle, 1975). The authors discussed, that the stimulation is consistent with the finding of an operative ribulose bisphosphate pathway of carbon assimilation in this bacterium. The same interpretation may apply to *R. acidophila* which like-

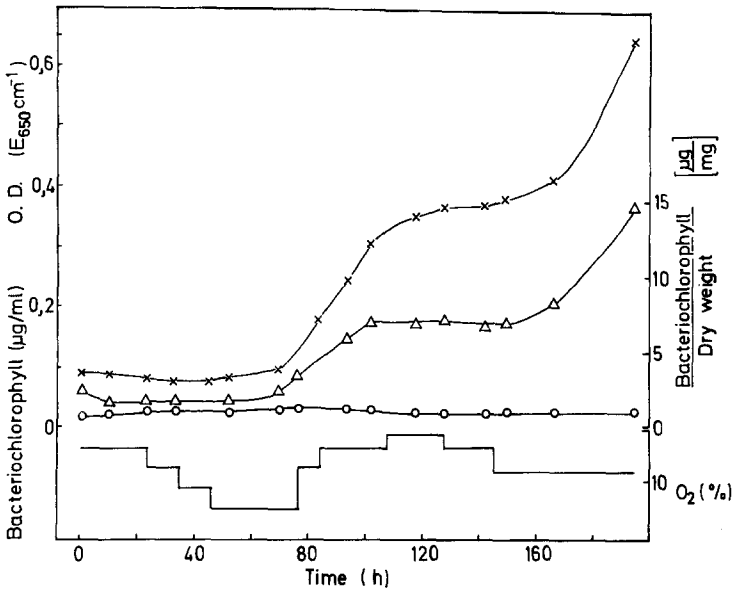

Fig. 2
Inhibition of chemolithoautotrophic growth of *Rhodopseudomonas capsulata* Kb1 by oxygen. The fermenter was inoculated with 500 ml of aerobically on succinate grown cells. Optical density (×——×). Bacteriochlorophyll in µg per ml (△——△). Bacteriochlorophyll per dry weight (○——○). Oxygen concentration of the gas phase (solid line)

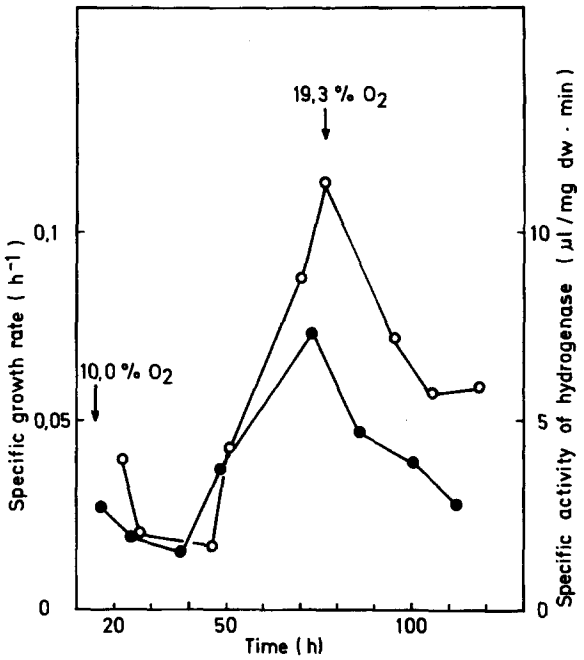

Fig. 3. Effect of change in oxygen concentration on specific hydrogenase activity and growth rate of chemolithoautotrophically growing cells of *Rhodopseudomonas capsulata* Kb1. The data were obtained from a batch culture experiment. Inoculum as in Fig. 1. Specific growth rate (●——●). Specific activity of hydrogenase (○——○)

wise does not contain the key enzymes of a reduced C_1-fixation sequence (Sahm et al., 1976).

A very low growth yield was obtained from aerobically growing cells of *R. acidophila* amounting to 23 g dry weight per 100 g methanol. Since carbon assimilation via the ribulose bisphosphate pathway has the highest energy requirement of all pathways found in one-carbon utilizers (van Dijken and Harder, 1975; Harder and van Dijken, 1976; Anthony, 1978), the low growth yield is consistent with the fact that this pathway of carbon assimilation is operative in *R. acidophila* when growing on methanol.

The rates of dehydrogenations of methanol, formaldehyde and formate have been suggested to limit growth rate rather than the rate of CO_2-fixation in *R. acidophila* growing anaerobically in the light on methanol (Douthit and Pfennig, 1976). The following calculation strongly supports this suggestion also for aerobic growth of *R. acidophila*. From the yield (y) on methanol and the corresponding specific growth rate (µ) the specific rate of methanol utilization can be estimated similar as described in "Material and Methods" for CO_2-fixation. *R. acidophila* growing anaerobically in the light on methanol has a maximum growth rate of 0.082 h^{-1} (unpublished results) and *y* was determined to be 0.98 g dry weight per g methanol (Quayle and Pfennig, 1975). From these values a specific rate of methanol utilization of 0.084 g methanol · g^{-1} dry weight · h^{-1} is obtained. For aerobically growing cells a virtually identical rate amounting to 0.087 g methanol · g^{-1} dry weight · h^{-1} is calculated using *y* and µ of aerobically growing cells as presented above. The 75% decrease of the specific growth rate under aerobic conditions thus turned out to be the consequence of a corresponding 75% decrease in growth yield rather than a change in activity of methanol dehydrogenation.

R. acidophila tolerated a relatively high dissolved oxygen tension (120 Torr = 16.0 kPa) for aerobic growth on methanol. In contrast, chemoautotrophic growth with H_2 as electron donor was completely inhibited above an oxygen tension of 25 Torr

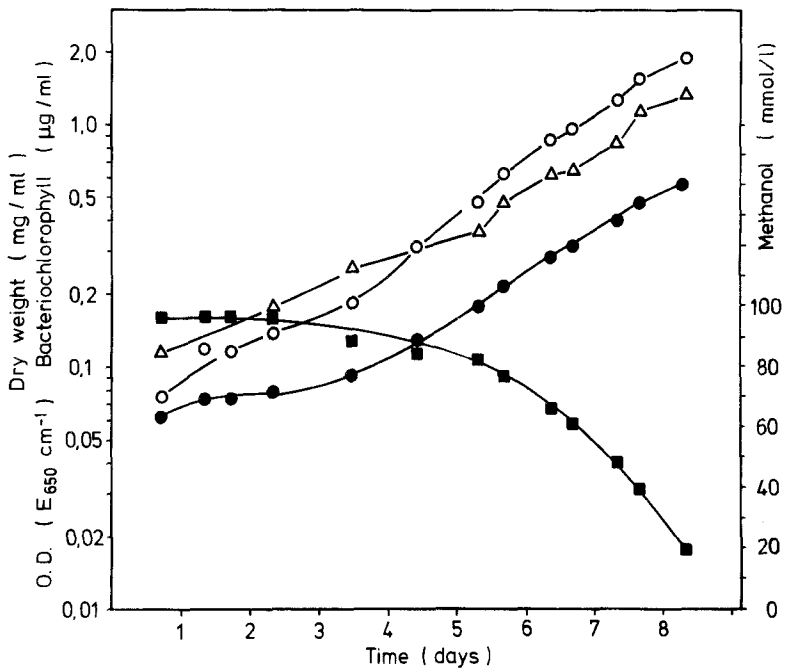

Fig. 4
Semiaerobic growth of *Rhodopseudomonas acidophila* 10050 on methanol and CO_2 in the dark. The growth experiment was carried out in a 2-l fermenter vessel inoculated with 100 ml of a culture grown anaerobically in the light on methanol. The gas phase contained 5% CO_2. The dissolved oxygen tension was maintained constant at 120 Torr (= 16.0 kPa) by means of an oxygen control device. Optical density (○——○). Bacteriochlorophyll in µg per ml (△——△). Dry weight in mg per ml (●——●). Methanol concentration in mmol per l (■——■)

(= 33.3 kPa). Growth with formate as energy and carbon source was most oxygen sensitive. Inhibition was observed already above an oxygen tension of 10 Torr (= 1.3 kPa). Although carbon assimilation in all three cases probably proceeds via the ribulose bisphosphate cycle, marked differences in respect to oxygen tolerance exist for this bacterium. It is, therefore, likely to conclude that oxygen sensitivity is due to enzymes involved in the dissimilation rather than assimilation of the supplied substrates.

Concluding Remarks

The capacity of resting cells of purple nonsulfur bacteria to catalyze the "Knallgas" reaction was first discovered by Gaffron (1935). This observation was later confirmed by other authors (Nakamura, 1938; Klemme, 1967; Meyer et al., 1978); they all examined the "Knallgas" reaction with resting cells, the capacity for growth under these conditions has not been reported. This could have been due to the application of unfavorable growth conditions or to an obligate light dependent electron flow for NAD reduction in the case of hydrogen. The latter possibility was suggested by experiments with cell-free extracts of *R. capsulata* in which the reduction of NAD by H_2 was light-dependent (Klemme and Schlegel, 1967). Later, an ATP-dependent reversed electron flow from H_2 to NAD was observed (Klemme, 1969) which proceeded with about 20% of the rate reported for the light-dependent reaction. The capacity of *R. capsulata* for chemoautotrophic growth indicates that the reduction of NAD by H_2 in the dark is indeed physiologically significant for this bacterium. There is convincing evidence now (Knaff, 1978) that this mechanism is operative even under anaerobic conditions in the light as first suggested by Gest (1963). We conclude, therefore, that the role of light in purple nonsulfur bacteria is restricted to photophosphorylation. Any substrate which will support respiratory energy conservation should also support chemotrophic growth.

Van Niel (1944) expressed the view that substrates which allow phototrophic development of facultatively aerobic purple nonsulfur bacteria, will also be utilized for aerobic growth in the dark. The results of the present paper confirm this statement for growth on H_2, formate and methanol provided the dissolved oxygen tension was kept low and an appropriate CO_2-concentration was supplied. Oxygen sensitivity of growth was observed also with other substrates. *R. acidophila* and *R. capsulata* did not tolerate the oxygen tension of air when alanine or aspartate respectively, was the energy source. Moreover, citrate which was described to support growth of *R. gelatinosa* only under anaerobic conditions in the light (Schaab et al., 1972) is utilized by this bacterium in the dark at low oxygen tension (unpublished results). It is likely, therefore, that in cases where substrates appear to be utilized only in the light by facultatively aerobic phototrophic bacteria, the proper chemotrophic growth conditions have not yet been established.

Note Added in Proof

The capacity for chemoautotrophic growth (85% H_2, 10% O_2, 5% CO_2) was recently shown for *Rhodopseudomonas capsulata* strain B 10

by M. T. Madigan and H. Gest: Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H_2 as energy source. J. Bacteriol. **137**, 524–530 (1979)

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