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Anaerobic Growth of Purple Nonsulfur Bacteria Under Dark Conditions

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Purple nonsulfur photosynthetic bacteria were cultured anaerobically in the absence of light by a modification of the Hungate technique. Growth was slow and resembled that of fastidious anaerobes; on yeast extract-peptone-agar medium, each cell produced about 16 descendants in 15 to 20 days. Growth was stimulated by addition of ethyl alcohol, acetate and H_2, or pyruvate and H_2. Cells grown in the presence of pyruvate and H_2 produced acetate and CO_2; each cell produced approximately 10 descendants in 24 hr under anaerobic, dark conditions. Spectrophotometric evidence obtained from cells which were the product of five generations suggests no difference between the bacteriochlorophyll and carotenoids synthesized by cells grown anaerobically under dark or light conditions. Likewise, the ultrastructure of the photosynthetic apparatus in cells grown anaerobically in the dark and in the light appears similar.

The ability of photosynthetic bacteria to grow in an anaerobic environment with dependence upon radiant energy was documented in a review by van Niel in 1944 (37). In the studies described, growth did not occur under identical anaerobic conditions in the dark. Recently, Pfennig (25) summarized knowledge of the photosynthetic bacteria and emphasized the anaerobic nature of their ecological niche. Yet, it is not unusual to find photosynthetic bacteria in anaerobic environments which are inaccessible to radiant energy, such as sewage sludge or layers of black mud from streams, as noted by Kandratieva (17); little is known of their persistence under such conditions. Certain nonsulfur purple bacteria have been shown to ferment substrates anaerobically in the dark (16, 21-23), and fructose was observed to stimulate the formation of colonies of *Rhodospirillum rubrum* on the surface of a solid growth medium (29). Nevertheless, the growth of photosynthetic bacteria in the dark under controlled anaerobic conditions has never been demonstrated.

In the present study, a pink-red colony was observed during the isolation of methane bacteria from anaerobic mud by the Hungate technique (4, 14). This organism proved to be a nonsulfur purple photosynthetic organism which grew slowly in the dark under conditions being used for the cultivation of methane bacteria. We have found that other representatives of the purple nonsulfur bacteria do indeed grow slowly under strictly anaerobic conditions in the absence of radiant energy. In this communication, we define the conditions under which growth occurs and present evidence that the membrane system of dark-grown cells is similar to that of light-grown cells.

MATERIALS AND METHODS

Bacteria. *R. rubrum* strain S_1 was obtained from H. Gest. *R. rubrum* strain 4 (Giesbrecht) and *Rhodopseudomonas viridis* NHTC 133 were obtained from C. Sybesma. *Rhodopseudomonas spheroides* strain 2.4.1 was obtained from S. Kaplan, and *Rhodopseudomonas palustris* was isolated in this laboratory from an anaerobic enrichment; N. Pfennig kindly identified this isolate.

Growth media. Cells were grown on a solid medium consisting of (grams per 100 ml distilled water): yeast extract (Difco), 0.3; Bacto-peptone (Difco), 0.3; agar, 2.0; and resazurin (Allied Chemicals, New York, N.Y.), as a redox indicator, 0.01. The mineral solution of Pfennig and Lippert (27) and a vitamin solution (39) also were added in 0.1- and 0.8-ml amounts, respectively, to 100 ml of growth medium. The cysteine-sulfide reducing agent of Bryant and Robinson (4) was sterilized and added separately to the growth medium. Liquid media of the same composition but without agar and supplemented with ethyl alcohol, sodium acetate, or sodium pyruvate (final concentration, 0.3 g per 100 ml of medium) were prepared. Ethyl alcohol and sodium pyruvate were filter-sterilized and added separately to the growth medium. Growth media were prepared and sterilized under a strictly anaerobic argon atmosphere by a modification of the Hungate technique (14) as described by Bryant

and Robinson (4). Sodium carbonate (0.2 g per 100 ml of growth medium) was added to media when gas mixtures which contained 20 or 50% CO_2 were used, and sodium phosphate buffer (*p*H 7.0) at a final concentration of 0.01 M was added to liquid growth medium which contained sodium pyruvate. The final *p*H of the sterile media was 6.9 to 7.1. For aerobic growth of cultures in the dark, liquid media of the same composition were prepared by omitting agar, resazurin, and cysteine-sulfide reducing agent.

Growth conditions. A simple modification of the Hungate technique was developed to maintain and grow cells under strictly anaerobic conditions. Sterile agar medium (25 ml) was placed into a 150-ml prescription bottle under a stream of argon. The bottle was aseptically sealed with a solid black-rubber stopper, and the agar was allowed to solidify on the flat side of the bottle. Cells were transferred aseptically with a platinum loop into the anaerobic bottle, and were spread over the agar by means of a glass rod (Fig. 1A) while a stream of O_2-free, sterile gas was passed into the bottle. Finally, the bottle was tightly sealed with a solid black-rubber stopper (Fig. 1B), and the neck was dipped into melted paraffin. Aseptic technique and manipulation of gas probes were performed as described previously (3,4). When gas mixtures which contained CO_2 were used, the growth medium was allowed to equilibrate with CO_2 before cells were spread onto the agar surface. Liquid media used in studies of growth under either anaerobic, light or anaerobic, dark conditions were prepared in a similar manner and dispensed into sterile test tubes as described by Bryant and Burkey (2). For certain purposes, cells were grown anaerobically under low light conditions (55 to 60 ft-c) obtained with a 60-w incandescent light bulb placed approximately 45 cm from the growth bottles or tubes. Dark-growth conditions were maintained by placing anaerobic bottles or test tubes in fiberboard (3 mm thick) cylinders with light-proof closures. Similarly, flasks of liquid media for aerobic, dark growth of cells were placed in a fiberboard cylinder which was fitted onto the table of a rotary shaker (New Brunswick Scientific Co. model VS-100). Each Erlenmeyer flask was filled to 30% capacity and was shaken at about 300 rev/min. Unless stated otherwise, cells to be used for inocula were serially subcultured at least twice under the specific conditions of the proposed experiment before an inoculum was taken for a growth experiment under the same conditions. The growth temperature in experiments was 34 to 37 C.

Preparation of gases. Gases used to maintain anaerobic environments were vigorously scrubbed to remove all traces of oxygen. Argon-CO_2 gas mixtures were prepared by means of a flow meter (SHO-RATE "dual-flow," Brooks Instrument Division, Hatfield, Pa.) with a common outlet. High-purity Ar, H_2, and H_2-CO_2 (80:20 or 50:50 mixture) gases were purchased from Matheson Gas Products, Inc. (Joliet, Ill.). Carbon dioxide was obtained from Simpson Distributing Co., Urbana, Ill. Trace amounts of O_2 were removed by passing the gases over hot reduced copper filings as described by Bryant, McBride, and Wolfe (3). In these experiments, two furnaces were connected in series. In addition, for certain experiments the gases were bubbled through a photochemically reduced methylviologen solution at *p*H 10 (31) as modified by Yu and Wolin (40). This additional precaution was taken to detect as well as to remove trace amounts of oxygen.

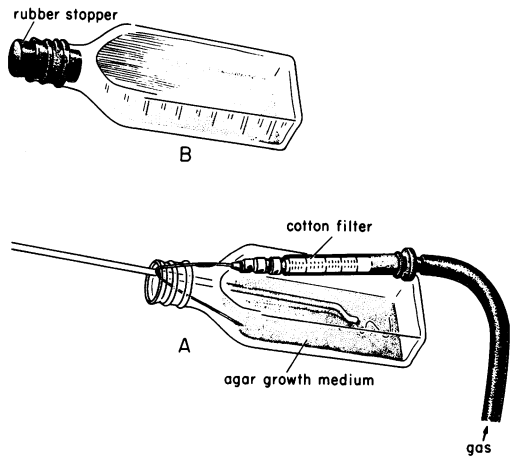

FIG. 1. *Schematic representation of technique used for growth of bacteria on an agar surface under strict anaerobic conditions: (A) inoculation of growth medium under continuous gassing; (B) rubber-stoppered growth bottle.*

Determination of cell numbers. Cell growth was washed from the surface of solid growth media and collected into a known volume. Samples of these cells and cells grown in liquid growth medium were counted directly by means of a Petroff-Hausser bacteria-counting chamber.

Protein. Samples were dried in vacuo and digested at 37 C for 1.5 hr in 1 N NaOH. Protein content was estimated by the method of Lowry et al. (19). Crystalline bovine serum albumin was used as a protein standard.

Bacteriochlorophyll. An acetone-methanol (7:2, v/v) solvent system was used to extract bacteriochlorophyll from washed cells (7), and the optical density was measured at 775 nm with a Beckman model DU spectrophotometer. The bacteriochlorophyll content in solvent extracts was estimated according to the method of Cohen-Bazire, Sistrom, and Stanier (7).

Gas chromatography. Carbon dioxide was detected with a Packard gas chromatograph equipped with a silica gel column connected to an argon ionization detector. Volatile fatty acids were resolved by placing acidified samples of culture supernatant liquid onto a Chromosorb W (60/80 mesh), acid-washed, 20% Tween 80 and 2% H_3PO_4 gas chromatographic column as described by Erwin et al. (11).

Spectroscopy. The detection and characterization of bacteriochlorophyll and carotenoids were accomplished spectrophotometrically with whole-cell suspensions in 60% sucrose solution as described by Pfennig (26). Spectra were obtained by use of a Cary 14 recording spectrophotometer.

Measurements of light-induced absorbancy changes in washed whole cells were obtained with a split-beam absorption difference spectrophotometer as described

by Sybesma and Fowler (33). Cells were suspended in the salts solution used by these investigators.

Photomicroscopy. Photomicrographs were taken with a flash attachment to a Zeiss photomicroscope.

Electron microscopy. Cells were fixed by the procedure of Kellenberger, Ryter, and Séchaud (15). Cells grown anaerobically in the dark were fixed with a 1% (w/v) solution of osmium tetroxide for 3 hr. Agar blocks were dehydrated in aqueous ethyl alcohol as described by Holt and Canale-Parola (13), and were embedded in Epon 812 by the method of Luft (20). Sections were cut on a Reichert ultramicrotome with a diamond knife and were mounted on uncoated 300- or 400-mesh copper grids. Sections were stained with a 1% solution of uranyl acetate for 45 min at 45 C (J. Murphy, *personal communication*) and with Pb-citrate, prepared according to the method of Venable and Goggeshall (38), for 6 min. All sections were examined with a Siemens Elmiskop I electron microscope operating at 60 kv, equipped with a 50-μm objective aperture.

RESULTS AND DISCUSSION

Rhodopseudomonas spheroides, *R. palustris*, *R. viridis*, and two strains of *Rhodospirillum rubrum* were grown under strictly anaerobic conditions in the dark. A typical culture of *R. rubrum* grown anaerobically in the dark, on the surface of the unsupplemented agar medium, is presented in Fig. 2. The organism was grown under a 20% CO_2-80% H_2 atmosphere; however, similar growth was readily obtained under an argon atmosphere. The use of bottle cultures (Fig. 1 and 2) has the advantages of the streak-plate for pure culture isolation, as well as the advantages of cell yields obtained through heavy inoculation and subsequent confluent growth. Furthermore, additional redox indicators may be included within the bottle but not in contact with the medium, as described below.

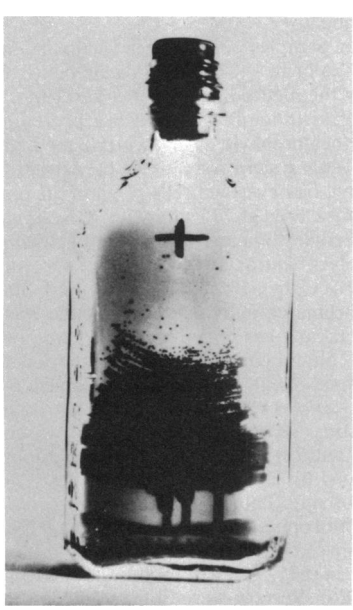

FIG. 2. *Photograph of anaerobic bottle showing anaerobic, dark growth of R. rubrum S_1. Cells were grown under a CO_2 (20%)-H_2 (80%) atmosphete at 37 C for 30 days.*

To establish that an anaerobic environment was maintained throughout our growth studies, the gas used during preparation of media and inoculation (Fig. 1) of the anaerobic bottles was extensively cleansed of oxygen, and resazurin was placed in the growth medium. Throughout the medium preparation and bacterial growth period, the medium remained colorless. Within seconds after exposure to air, the resazurin became oxidized, as indicated by a color change to pink.

Additional evidence was sought to demonstrate that anaerobiosis was obtained and maintained by this technique. An alkaline solution of methylene blue (0.1%, w/v) in 0.5% glucose (w/v) was boiled under a stream of O_2-free gas until colorless, and 10 ml of the reduced solution was added anaerobically to a growth bottle of *R. palustris* which had been placed on its side, agar side up. In this manner, the reduced methylene blue solution had a large surface exposed to the gas atmosphere but did not contact the agar medium. After 10 days of incubation in the dark, bacterial growth was apparent on the agar; the indicators remained colorless. Upon exposure to air, the methylene blue solution turned blue and the resazurin in the growth medium turned pink. *R. spheroides* and *R. palustris* grew under identical dark conditions when the methylene blue indicator solution was replaced with 10 ml of a slightly turbid culture of *Escherichia coli* in Nutrient Broth (Difco). After 10 days, typical growth of the photosynthetic organisms had developed on the surface of the solid medium. Upon exposure to air, the resazurin contained in the solid yeast extract-peptone medium turned pink, and *E. coli* in the broth grew to a high density (10^9 cells/ml). It was concluded from these experiments that strictly anaerobic conditions were maintained in the culture bottles during the growth of purple nonsulfur bacteria. Moreover, the same anaerobic bottle technique supported growth of the strictly anaerobic, methane-producing bacteria *Methanosarcina* and *Methanobacterium* strain M.o.H. Growth media have been maintained in a reduced state under these conditions for several months, and *Methanosarcina* remained viable for 7 months in an unopened bottle with methanol as substrate, indicating the absolute anaerobic state of the culture conditions.

To show that no light was available during growth, photographic plates were attached to the inside of the fiberboard cylinders; these plates failed to become exposed. Dark growth of the purple nonsulfur bacteria was unaffected when infrared radiation was excluded by wrapping the growth bottles or the fiberboard cylinder, or both, with heavy duty aluminum foil.

On the unsupplemented agar medium, *R. rubrum*, *R. palustris*, and *R. spheroides* showed growth in heavily inoculated areas 10 days after inoculation, and individual colonies about 1 mm in diameter developed by 20 days. Growth was not stimulated on this medium by replacing the Ar atmosphere with H_2, CO_2, or gas mixtures containing 20% CO_2 and 80% H_2 or Ar. Growth and motility could be maintained by repeated transfer of each organism under dark, anaerobic conditions. Cells maintained under dark conditions were transferred quickly in a semidark room.

The growth of *R. rubrum* S_1 and *R. rubrum* 4 under anaerobic, dark conditions was determined by direct cell count. A cell suspension of young cells grown anaerobically in the dark was prepared, and a 0.1-ml sample was spread onto the surface of agar growth medium with a glass spreader. The argon-filled bottles were incubated, agar side up, in the dark. After 23 days, cell growth was determined (Table 1). Motile cells were observed under phase-contrast microscopy (Fig. 9). Both strains of *R. rubrum* grew at a very slow rate, requiring approximately 5 days for one generation. When experiments were conducted with *R. palustris*, similar growth occurred, but an accurate cell count was not obtained as the cells formed clumps which could not be dispersed. Cells of the purple nonsulfur bacteria investigated possessed good pigmentation after growth under anaerobic, dark conditions.

Kohlmiller and Gest (16) and Nakamura (21–23) reported that under dark, anaerobic conditions *R. rubrum* S_1 and *R. palustris* fermented certain organic acids. It is conceivable that the same fermentative pathways are functional in cells growing anaerobically in the dark. Cells of *R. rubrum* S_1 were placed into the liquid yeast extract-peptone growth medium to which various short-chain fatty acids, alcohols, and dicarboxylic acids were added singly or in combination. It was found that growth under anaerobic, dark conditions could be significantly stimulated by the addition of ethyl alcohol under an Ar atmosphere and acetate or pyruvate under an H_2 atmosphere (Table 2). The best cell growth obtained after 10 days at 37 C with ethyl alcohol or acetate was 2.30×10^8 and 2.24×10^8 cells/ml, respectively. Pyruvate stimulated the growth of *R. rubrum* which, after 36 hr at 37 C, reached approximately 2×10^8 cells/ml. The addition of pyruvate reduced the time required for one generation from approximately 5 days on yeast extract-peptone-agar growth medium to 8.6 hr in liquid medium (Tables 1 and 2). The growth of *R. rubrum* S_1 under anaerobic, dark conditions in broth with pyruvate is shown as an increase in protein and bacteriochlorophyll in Fig. 3 and 4. Figure 3 presents the protein increase of cells grown for 72 hr. In this experiment, *R. rubrum* S_1 cells were grown anaerobically in the light in the presence of pyruvate for 24 hr and were then placed into fresh medium with pyruvate under anaerobic conditions in the dark. There was no lag in protein synthesis when the cells were transferred from light to dark growth conditions. During the first 6 hr after transfer to a dark environment, the bacteriochlorophyll content decreased, and then it increased parallel with protein synthesis. Protein synthesis by cells subcultured serially twice in a dark environment is presented in Fig. 4. Again, no lag in protein synthesis was observed, and bacteriochlorophyll increased proportionately. The growth rate of cells passed from anaerobic, light to anaerobic, dark conditions (Fig. 3) was greater than that of cells passed from anaerobic, dark to anaerobic, dark growth conditions. The rate of growth was sig-

TABLE 1. *Growth of Rhodospirillum rubrum under anaerobic, dark conditions on yeast extract-peptone-agar medium*[a]

Strain	No. of cells		No. of generations[b]
	Initial (N_0)	Final (N_1)	
S_1	4.2×10^7	107×10^7	4.7
4	17.6×10^7	345×10^7	4.3

[a] Cells grown under Argon atmosphere for 23 days at 37 C.

[b] Number of generations = $\log_2 N_1 - \log_2 N_0$ where N represents the number of cells.

TABLE 2. *Growth of Rhodospirillum rubrum S_1 on liquid medium under anaerobic, dark conditions*

Substrate	Gas phase	Period of incubation (hr)	No. of cells		No. of generations
			Initial (N_0)	Final (N_1)	
Ethyl alcohol	Argon	240	0.68×10^7	23.0×10^7	4.75
Acetate	Hydrogen	240	0.48×10^7	22.4×10^7	5.54
Pyruvate	Hydrogen	36	1.30×10^7	23.4×10^7	4.17

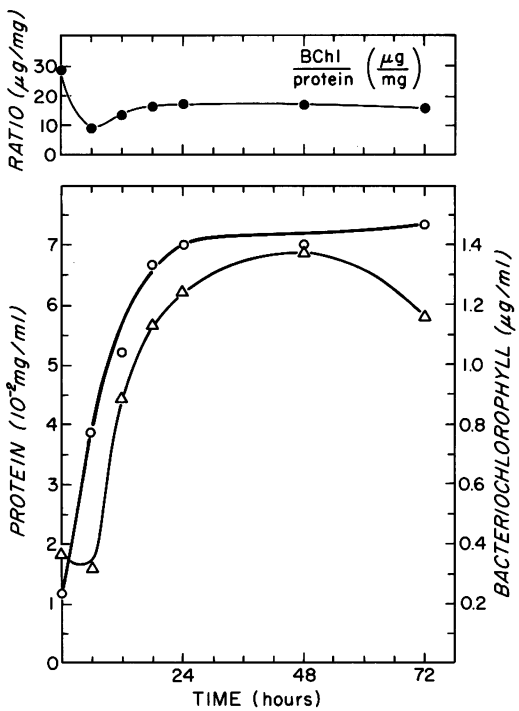

FIG. 3. *Increase of protein* (○) *and the bacteriochlorophyll* (△) *content in R. rubrum* S_1 *cells grown on pyruvate in the light anaerobically and transferred into dark, anaerobic conditions. Bacteriochlorophyll concentration as a function of protein synthesis is presented above* (●).

nificantly increased under an atmosphere of H_2 by the addition of pyruvate (Tables 1 and 2). Schoen (29) observed that *R. rubrum* formed colonies in the dark under anaerobic conditions if fructose was present in the growth medium. We observed no stimulation of the anaerobic, dark growth of *R. rubrum* S_1 in the presence of glucose, fructose, or glycerol.

Kohlmiller and Gest (16) reported that *R. rubrum* S_1 cells grown under both anaerobic, light and aerobic, dark conditions fermented pyruvate anaerobically in the dark and produced CO_2, acetate, and propionate. In our experiments, *R. rubrum* S_1 cells grown under dark, anaerobic conditions (5 to 15 generations) produced only CO_2 and acetate. In our system, anaerobic, dark growth of *R. rubrum* on pyruvate under a H_2-CO_2 (50:50) gas phase failed to form propionate. Trace amounts of propionate were detected only in the supernatant solutions of cultures grown under light, anaerobic conditions. The fermentation of pyruvate during dark, anaerobic growth resulted in a drop in the *p*H value of the medium from 7.0 to 5.7 within 48 hr after inoculation. This might account for the observation that, after anaerobic, dark growth on pyruvate for 48 hr at 37 C, *R. rubrum* S_1 cells rapidly became nonphototactic and lost viability. Consequently, to maintain cells under anaerobic, dark growth conditions they were transferred every 36 hr into fresh growth medium.

Bacteriochlorophyll-a was extracted from cells of *R. rubrum* S_1 described in Table 1. The bacteriochlorophyll-a content in the solvent extract (7) and the protein content remaining in the cell debris were determined (Table 3). The bacteriochlorophyll-protein ratios of strains S_1 and 4 are different; the significance of this difference is not known. The bacteriochlorophyll-protein ratio for

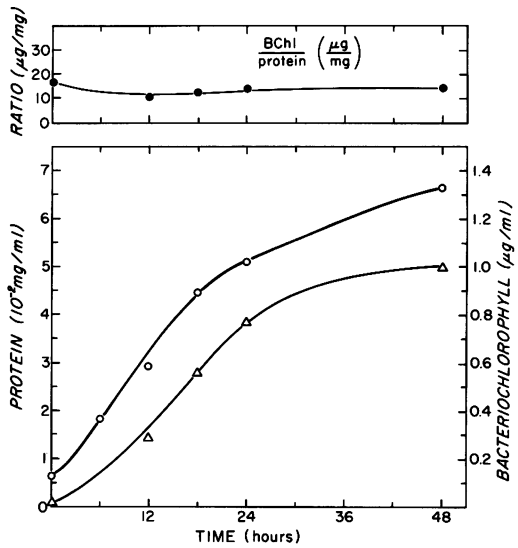

FIG. 4. *Increase of protein* (○) *and bacteriochlorophyll* (△) *in R. rubrum* S_1 *cells grown on pyruvate, under dark, anaerobic conditions. Bacteriochlorophyll synthesis as a function of protein increase is presented above* (●).

TABLE 3. *Bacteriochlorophyll produced by R. rubrum grown under anaerobic, dark conditions*[a]

Strain	Bacteriochlorophyll[b]	Protein[c]	Bacteriochlorophyll/mg of protein
	μg	mg	μg
S_1	1.28	0.256	14.0
4	8.00	0.573	5.00

[a] Cells grown for 23 days at 37 C.

[b] Corrected for bacteriochlorophyll content in inoculum. (S_1 = 2.75 μg; strain 4 = 2.26 μg under anaerobic, light conditions)

[c] Contained in cell residue after extraction of bacteriochlorophyll.

R. rubrum S_1 is similar to that obtained by Schick and Drews (28) for cells placed under semi-aerobic, dark conditions, and is significantly less than that obtained with *R. rubrum* grown anaerobically under low-intensity light (6, 18, 30).

The ratio of bacteriochlorophyll to protein in *R. rubrum* S_1 cells grown anaerobically under low-intensity light immediately decreased in the absence of light and stabilized at a new lower value (Fig. 3 and 4).

Whole cells of *R. palustris* which had been subcultured at least once under anaerobic, dark conditions were suspended in a 60% sucrose solution (w/w) and were observed spectrophotometrically. Figure 5 presents a comparison of the light absorption spectra of 5-day-old cells grown aerobically in the dark, anaerobically in the dark, and anaerobically in the light. As noted by previous investigators (5–6, 8, 18, 35), absorption maxima typical of bacteriochlorophyll or carotenoids were not observed in cells grown aerobically in the dark. The spectra obtained from cells of *R. palustris* grown anaerobically under dark or light conditions were identical, with bacteriochlorophyll-a maxima at 375 (not pictured), 597, 803, 854, and 890 (shoulder) nm; characteristic carotenoid absorption maxima were observed at 469, 499, and 532 nm, as noted by Pfennig (25, 26; personal communication) and Cohen-Bazire et al. (7). Figure 6 shows the results of a spectrophotometric study of *R. rubrum* S_1 grown under similar conditions. Cells grown aerobically in the dark show no characteristic absorption maxima, and cells grown anaerobically under both light and dark conditions show identical absorption maxima for bacteriochlorophyll-a at 588, 801, and 882 nm and for carotenoids at 484, 511, and 548 nm (7).

R. viridis (10, 24) was grown anaerobically under light and dark conditions on the yeast extract-peptone-agar medium previously described to which sodium malate (0.3 g per 100 ml of medium) was added. The spectra obtained from whole cells grown under both conditions were similar (Fig. 7), with absorption maxima at 397 (not pictured), 605, 825, and 1,015 nm, which are typical for bacteriochlorophyll-b (9, 12, 24). The remaining absorption maxima are attributed to carotenoids, with the exception of the bacteriochlorophyll-b maximum at 684 nm in cells grown anaerobically in the light; the nature of this maximum is not understood (36).

Separate cultures of *R. rubrum* S_1, *R. palustris*, and *R. spheroides* 2.4.1 were allowed to grow anaerobically under dark conditions for approximately five generations before preparation for electron microscopy. The cells of each strain used for the inoculum had been maintained under

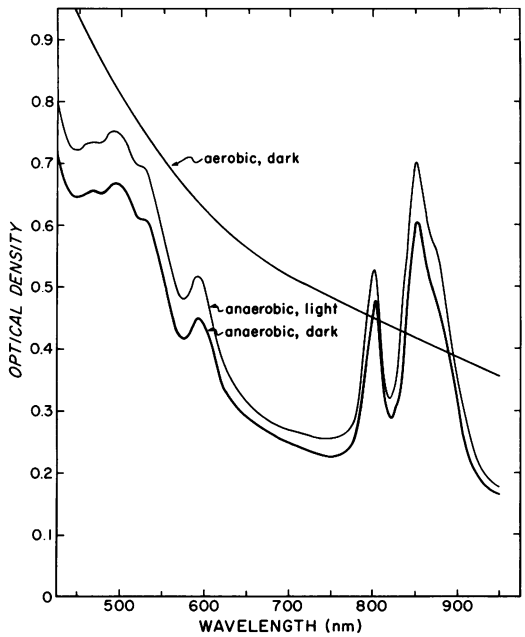

FIG. 5. *Absorption spectra of R. palustris. Cells were suspended in 60% (w/w) sucrose solution.*

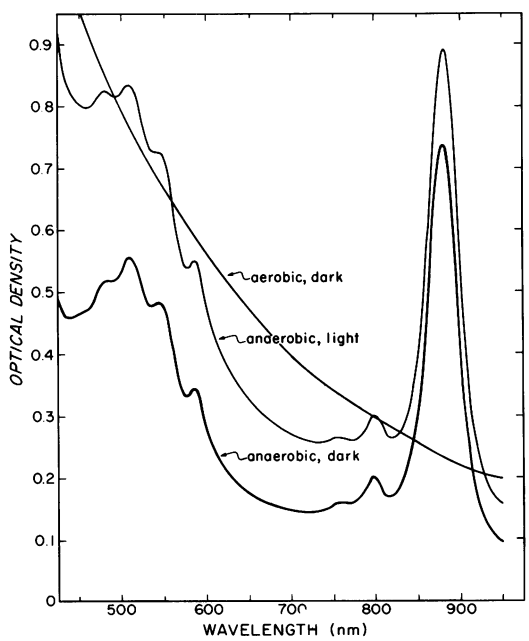

FIG. 6. *Absorption spectra of R. rubrum* S_1. *Cells were suspended in 60% (w/w) sucrose solution.*

anaerobic, dark growth conditions. Figure 9 is a phase-contrast photomicrograph of motile phototactic cells of *R. rubrum* S_1 which had been grown under anaerobic, dark conditions. For compara-

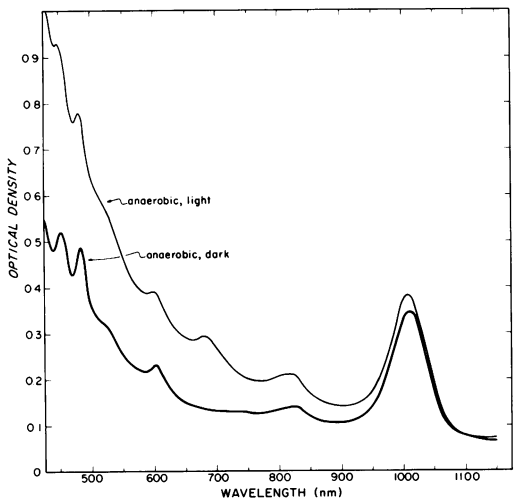

FIG. 7. *Absorption spectra of R. viridis NHTC 133. Cells were suspended in 60% (w/w) sucrose.*

tive purposes, cells grown anaerobically in the light also were prepared for cytological studies.

Electron micrographs of thin sections of *R. rubrum* S_1 cells grown under anaerobic, dark conditions (Fig. 8) appeared identical with those of cells grown under anaerobic, light conditions (Fig. 10). From our observations, "chromatophore" structures are distributed through the cell cytoplasm of cells grown anaerobically under dark conditions, as is also the case in cells grown under light conditions (Fig. 10). Biedermann et al. (1) and Drews and Giesbrecht (8) also found extensive "chromatophore" formation in cells of *R. rubrum* placed under semiaerobic, dark conditions.

Electron micrographs of sectioned cells of *R. spheroides* 2.4.1 grown anaerobically under dark and light conditions are presented in Fig. 11 and 12, respectively. Cells grown under anaerobic, dark conditions (Fig. 11) contained a membrane system of characteristic "chromatophore" structures. Membrane content was, however, less extensive than in cells grown under anaerobic, light conditions (Fig. 12). Figure 14 is an electron micrograph of a cross section through the lamellar membrane system of *R. palustris* formed in cells grown under anaerobic, dark conditions. The membrane network in these cells was less extensive than that in cells grown under anaerobic, light conditions (Fig. 13). Numerous cells grown anaerobically in the dark were fixed while in the process of cell membrane invagination, a process leading to the formation of the lamellar-type membrane apparatus (34) shown in Fig. 13 and 14. An example of this process is presented in Fig. 15.

No cytological distinctions were observed between fifth or sixth generation cells grown anaerobically under dark and light conditions. The dimensions of the unit membrane networks were not significantly different. It was not possible to ascertain this for *R. spheroides* because of poor fixation of the light-grown cells (Fig. 12).

Biedermann et al. (1) found that purple nonsulfur bacteria, which can respire aerobically, formed an apparently functional photoreceptive membrane network in the absence of, or with restricted amounts of, oxygen. Moreover, carotenoid- and bacteriochlorophyll-containing membrane structures also were synthesized in an environment where they serve no photoreceptive function. Results of chemical and cytological studies, as well as observations of phototaxis, indicate that these photoreceptive membranes are functional.

R. rubrum S_1 cells were grown for approximately 20 generations under dark, anaerobic conditions, and the light reactivity of the photosynthetic apparatus was observed spectrophotometrically. Figure 16 is a light minus dark difference spectrum of the photoactive P890 and P800 center (32). Likewise, the light minus dark difference spectrum of *R. rubrum* cytochromes C422 and C428 is presented in Fig. 17 (33). When cells grown anaerobically in the dark were exposed to an actinic light beam at 874 nm, the cytochromes became oxidized, as characterized by a decrease in absorption; when the actinic light was shut off, the absorption increased as the cytochromes became reduced. The "fast" decrease in absorption of cytochrome C428 oxidation and the "fast" increase in absorption of cytochrome C422 reduction is presented in Fig. 17. The same cells exhibited a rapid light-induced nicotinamide adenine dinucleotide reduction when observed by fluorometric techniques. These data indicate that cells of *R. rubrum* S_1 growing under anaerobic, dark conditions continued to synthesize a complete light-responsive photosynthetic apparatus.

So far, we have examined only members of the *Athiorhodaceae*, but there appears to be no reason why members of the *Thiorhodaceae* and *Chlorobacteriaceae* should not grow anaerobically in the dark. Since the photosynthetic mode of life for bacteria in nature is located in anaerobic environments (25), it could be of considerable advantage for a cell to have a preformed photoreceptive apparatus while in a transitory anaerobic, dark environment. Continued study of anaerobic, dark growth of photosynthetic bacteria may provide information helpful in under-

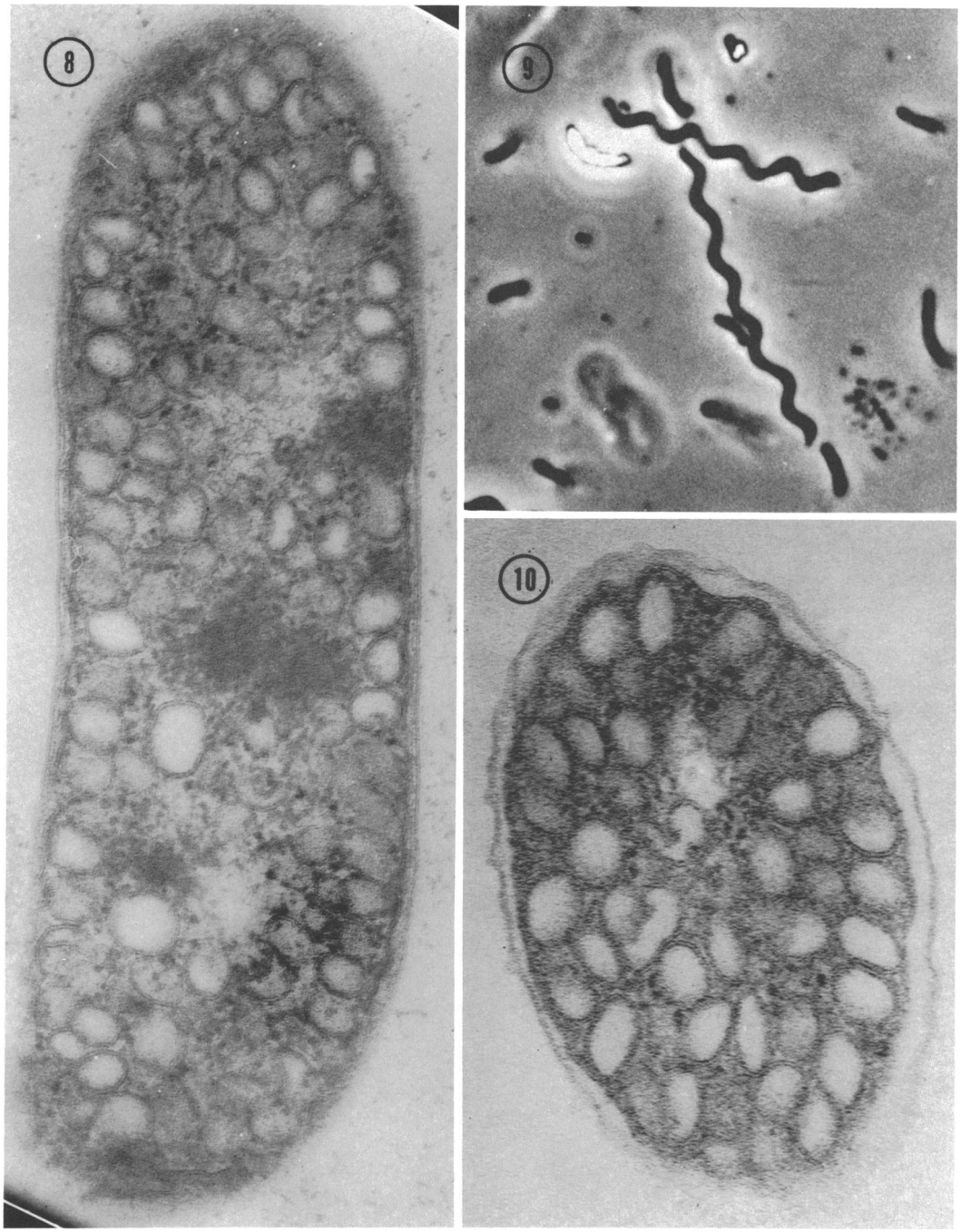

FIG. 8. *Electron micrograph of longitudinal thin section of R. rubrum S_1 grown under anaerobic, dark conditions. Unless otherwise noted, for electron micrographs the main fixation time was 3 hr and each section was poststained with uranyl acetate and lead citrate.* × *112,000.*

FIG. 9. *Phase-contrast photomicrograph of motile R. rubrum S_1 cells grown anaerobically in the dark.* × *1,900.*

FIG. 10. *Electron micrograph of thin transverse section of R. rubrum S_1 grown under anaerobic, light conditions.* × *104,000.*

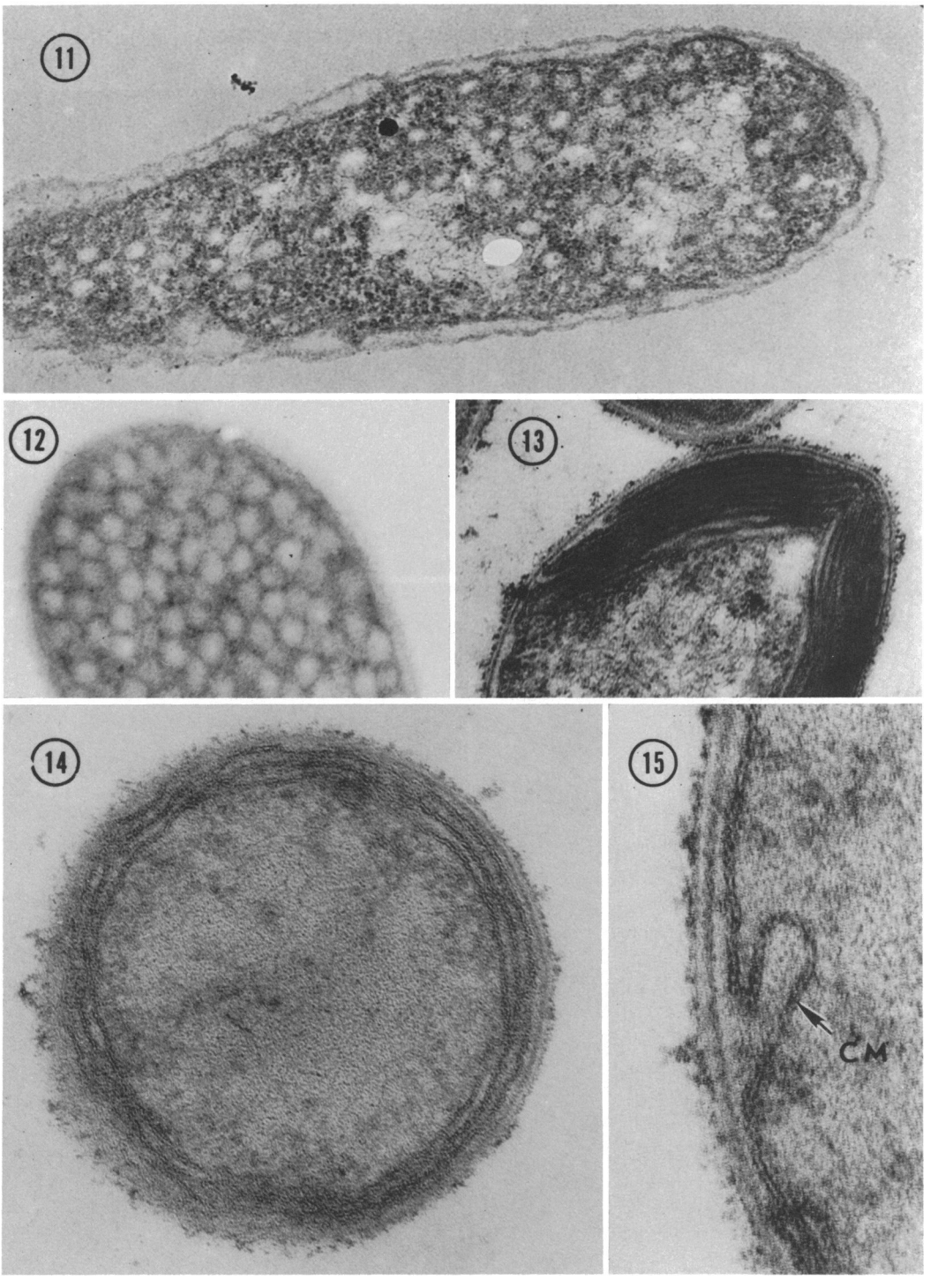

FIG. 11. *Electron micrograph of R. spheroides 2.4.1. Longitudinal thin section. Cell grown under anaerobic, dark conditions.* × *56,000.*

FIG. 12. *Electron micrograph of thin section of R. spheroides 2.4.1. Cell grown under anaerobic, light conditions.* × *52,000.*

FIG. 13. *Electron micrograph of thin transverse section of R. palustris. Cell grown under anaerobic, light conditions. Main fixation time was 11 hr; poststained with uranyl acetate and lead citrate.* × *71,400.*

FIG. 14. *Electron micrograph of thin transverse section of R. palustris grown under anaerobic, dark conditions.* × *115,000.*

FIG. 15. *Electron micrograph of thin section of R. palustris grown under anaerobic, dark conditions. Cell membrane (CM) in process of invagination to form "photosynthetic" apparatus.* × *207,000.*

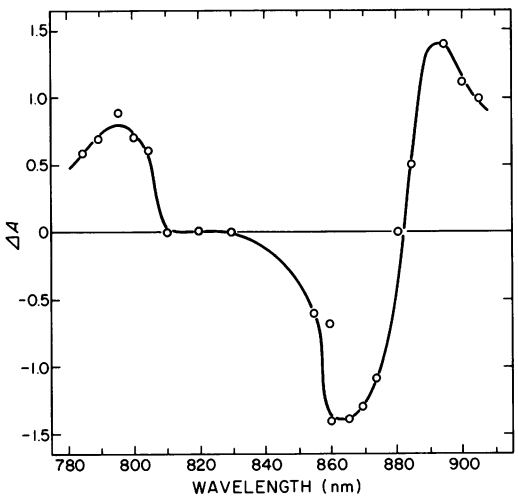

FIG. 16. *Light minus dark difference spectrum in the near infrared spectral region of R. rubrum S_1 cells grown anaerobically in the dark and induced by 586-nm actinic light.*

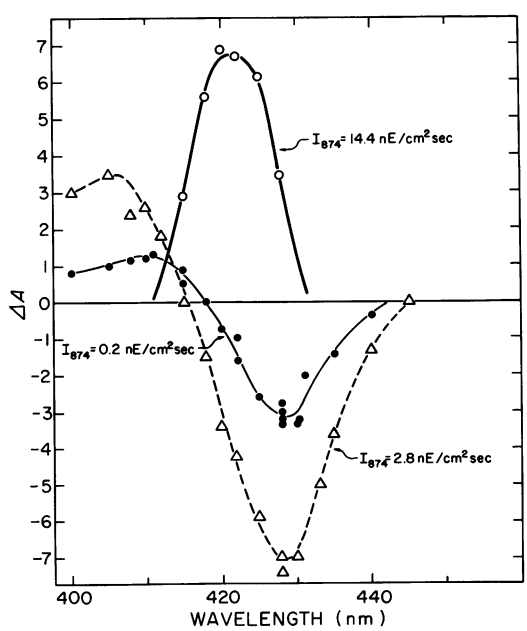

FIG. 17. *Light minus dark difference spectra of cytochromes in R. rubrum S_1 cells grown under anaerobic, dark conditions. Cells were suspended in mineral-salts solution. Cytochrome C428 light-induced absorbancy decrease at low (●) and medium (△) light intensities; 0.2 and 2.8 nE/cm² sec, respectively. Light induced response of cytochrome C422 (○) is presented as the fast absorbancy increase after shutting off the light. (I_{874} = actinic light beam at 874 nm.)*

standing the development of the photosynthetic apparatus and its functioning.

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